INTRAGASTRIC GELATION OF MIXED SOY PROTEIN ISOLATE AND ALGINATE AS
WELL AS ITS EFFECT ON POSTPRANDIAL GLUCOSE RESPONSE AND SATIETY

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INTRAGASTRIC GELATION OF MIXED SOY PROTEIN ISOLATE AND ALGINATE AS WELL AS ITS EFFECT ON POSTPRANDIAL GLUCOSE RESPONSE AND SATIETY

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ABSTRACT

The goal of the study is to investigate the effect of alginate on sucrose release and *in vitro* gastric digestion of soy protein isolate (SPI) in model beverages as well as to determine whether consumption of the model beverages would affect postprandial blood glucose response and appetite in healthy adults. Model beverages containing 5% w/v SPI, 0 to 0.20% w/v alginate and 10% w/v sucrose were prepared by heating the mixtures at 85 °C for 30 min at pH 6.0 or 7.0. Characterizations of beverages included determination of zeta-potential, particle size analysis and rheological properties measurement. Digestion patterns and sucrose release were determined after 0 to 2 h in-vitro gastric digestion using SDS-PAGE and HPLC analysis, respectively. Results showed that increasing alginate concentration led to increased zeta-potential value, particle size as well as increased viscosity and pseudoplastic behavior; however, no phase separation was observed in any of the samples. In the absence of alginate, the SPI beverage could form a weak intragastric gel only at a pH of 6.0 after mixing with simulated gastric fluid (SGF), while at pH 7.0 a gel was formed only in the presence of alginate. Formation of the intragastric gel led to delayed protein digestion and slower release of sucrose. Higher resistance to digestion and a slower sucrose release rate were exhibited at increased alginate concentration, and to a

lesser extent, at pH 6.0. This suggests that electrostatic interaction between SPI and alginate that occurred when the beverages were under gastric condition could be responsible for the intragastric gelation.

The hypothesis that beverages showing intragastric gel formation in the *in vitro* study could be translated into *in vivo* applications was tested in a clinical trial. In the clinical trial, after an overnight fast, twelve healthy subjects were asked to consume six standardized breakfast beverages in a randomized order: a 122 kcal sugar beverage (CONT), a 122 kcal sugar beverage with alginate (ALG), a 172 kcal sugar beverage with SPI at pH 7 (SPI-7) or pH 6 (SPI-6), a 172 kcal sugar beverage with mixed SPI and alginate at a pH 7 (SPI+ALG-7) or pH 6 (SPI+ALG-6). Subjects consumed one of the beverages at time 0. Blood samples were drawn at -15, 0, 15, 30, 45, 60, 90 and 120 min and questionnaires were completed immediately following the blood draw at each time point. Results showed that, compared to CONT, consumption of SPI-6, SPI+ALG-7 and SPI+ALG-6 significantly lowered peak blood glucose concentration and 1-h incremental area under the curve (AUC). SPI+ALG-6 also exhibited a significant reduction in 2-h AUC. No significant effect on appetite was found in any condition. Interactions between the protein and alginate during digestion and formation of an intragastric gel could play an important role in influencing postprandial blood glucose response.

In conclusion, we demonstrated the possible formation of an intragastric gel resulted from the SPI and alginate mixture under certain conditions, which subsequently delayed protein digestion and sucrose release from the matrix. Compared with CONT, consumption of beverages that formed an intragastric gel (SPI+ALG-7 and SPI+ALG-6) attenuated the postprandial glycemic concentration in healthy adult subjects. These results could potentially lead to the formulation of SPI beverage with functionality to lower postprandial glycemic response.

CHAPTER 1

INTRODUCTION

It is reported that globally there are 387 million people diagnosed with diabetes, and by 2030, diabetes is projected to be the 7th leading cause of death worldwide (Giovannini and others 2016). The prevalence of diabetes will continue to grow as the population ages and diets become more heavily sugar and fat-based. New research revealed that the nation spent a record-high of \$245 billion on diabetes-related programs in 2012, a 41% increase from \$174 billion in 2007 (American Diabetes Association 2013). An effective way to reduce the chance of acquiring diabetes and coronary heart disease is to reduce the dietary glycemic response (Livesey and others 2008). Both fasting glucose and postprandial plasma glucose concentration are directly correlated to the risk of diabetes complications; high postprandial glucose levels could potentially constitute a stronger risk factor for cardiovascular complications (Bonora and Muggeo 2001; Temelkova-Kurktschiev and others 2000; Monnier and others 2003). Glycemic control has an emphasis on fasting and postprandial glucose control. The glycemic index (GI) indicates the difference of postprandial glucose response after taking a specific type of food (Wolever and others 1991). It is defined as the net incremental area under the plasma glucose curve (e.g., area under the curve or AUC) for the food in question and expressed as a percentage of that of a standard control (Wolever 2004). While consuming low GI foods will lower postprandial glucose response and, in some studies, also sustains insulin secretion. With improved blood glucose control, the risk of developing complications associated with diabetes is significantly reduced (UKPDS Group 1998; O'Keefe and Bell 2007).

Soy protein is one of the most nutrient-rich among the many legume proteins due to their high protein level and well-balanced amino acid composition. Soy protein has been widely regarded as a key ingredient in food formulation to enhance nutrients, functionalities, and qualities. Soy protein-containing foods possess a number of health benefits, namely lowering the risk of acquiring heart disease and diabetes. With consumers' awareness of these benefits and the increasing popularity of protein-based drinks, the market of soy protein-containing foods, especially soy protein beverages, has been experiencing a sizable growth. It is proposed that dietary protein plays an active role in lowering the risk of diabetes complications by diminishing postprandial glucose, which is achieved through gastric emptying deceleration. Diabetes complications are possibly inhibited with the presence of soy proteins and may improve cardiovascular risk factors (Gannon and others 2003).

Dietary fibers had been shown to have positive effects on body weight and glycemic control (Babio and others 2010; Slavin 2005; Schulze and others 2004). Though insoluble fibers have been found to correlate with decreasing risk of type 2 diabetes and cardiovascular complications, the magnitude of their effects on postprandial glucose and insulin responses, LDL and total cholesterol are comparable to viscous soluble fibers (Weickert and Pfeiffer 2008). Viscous soluble fibers comprise a wide range of uses that include modifying the viscosity as well as improving texture and stability of foods and beverages. Soluble dietary fibers have been shown to be effective in regulating the postprandial glucose levels by altering food texture, structure, and viscosity (Brennan 2005). Bakalis and others (2007) used a human intestine model to demonstrate this mechanism: they employed a guar gum solution to increase food viscosity so that flow of material to the GI tract would slow down, which would eventually exert an impact on digestion and uptake of other nutrients. The mechanism in glycemic control is proposed to be

their ability to increase the viscosity of gastrointestinal digesta, resulting in slowing the digestion and preventing bulk diffusion of foods. The slow absorption leads to decelerated postprandial glucose and insulin responses, which prompt meaningful inferences in prevention and management of type 2 diabetes (Juntunen and others 2002).

The pattern of protein digestion in the gastrointestinal (GI) tract is highly dependable on several factors such as gastric conditions, physical properties of the protein, and the presence of other food components in the matrix. Various kinds of soluble fibers ranging from pectin, cellulose, and alginate that work as stabilizers and thickeners are popular ingredients in protein-based products to modify the viscosity as well as improve the texture and stability of food or beverages. Several articles have shown the interactions between soy protein and polysaccharides at low pH conditions (Yuan and others 2013; Lam and others 2007; Lam and others 2008); however, the effect of polysaccharides on soy protein digestion is not fully understood. Previous studies showed that mixed solutions of dairy proteins (whey and casein) and negatively charged polysaccharide formed intragastric gelation when the mixtures were added to simulated gastric fluid (SGF) (Zhang and others 2014c; Borreani and others 2016). The intragastric gelation system is believed to derive from the electrostatic interaction between positively charged residues on protein and negatively charged polysaccharides when pH was changed from near neutral to acidic under simulated gastric conditions. The protein degrading process significantly decelerated in an intragastric gel environment. Implications of this mechanism might include slowing down gastric emptying, inducing satiety and lowered sucrose release profiles. In contrast to regular liquid food, the transformation of protein-containing liquid (e.g., beverages) into intragastric gel would require a longer transit time in the stomach. Thus, it is highly possible that

the sucrose could be entrapped inside the intragastric gel and would take a longer time to be released, resulting in better postprandial glycemic control (Hur and others 2011).

The overall goal of this study was to systematically investigate the intragastric gelation and the release profile of sugar from soy protein isolate and alginate mixtures in beverages. Changes in sugar release from the in-vitro digestion study were further investigated by determining postprandial glucose response of selected treatments in healthy human subjects.

Specific objectives were: (i) to determine key factors that affect digestion behavior (e.g., digestion rate and intragastric gel-formation) of mixed soy protein and soluble fiber beverages under in vitro gastric digestion, (ii) to determine the effect of intragastric gelation of mixed protein-fibers on the release of sugar, and (iii) to determine whether consumption of beverages/snacks containing mixed soy protein and soluble fiber change digestion behavior that will result in differences in post-prandial sugar release in healthy subjects. The outcome of this study could have a positive impact on the soy protein market as well as improve public health.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Soy Protein and Its Health Benefits

2.1.1 Soy Protein

Isolated from dehulled and defatted soybeans, soy protein is a “whole protein” that furnishes complete essential amino acids for human nutrition. Due to its functionalities, soy protein isolate has long been used in commercial food products to enhance nutrient values and health benefits. As approved by the FDA, products containing soy protein are allowed to have the health claim with the following statement: “The addition of soy protein to a diet that is low in saturated fat and cholesterol may help to reduce the risk of coronary heart disease”. Other potential health benefits of soy protein include control of hyperglycemia and reduced body weight as well as prevention of cancer (Friedman and Brandon 2001; Singh and others 2008; Deibert and others 2004; Badger and others 2005). Moreover, as food-labeling policies across the globe approve health claims for soy protein-enriched foods, soy protein continues to gain popularity and becomes an even more favorable ingredient in the food industry.

2.1.2 Composition of Soy Protein

There are two main fractions of soy protein that represent more than 85% of the storage proteins in the seed, 7S globulin (β -Conglycinin) and 11S globulin (glycinin). The 7S globulin is a heterogeneous glycosylated trimeric glycoprotein consisting of at least six combinations of three subunits: α (76 kDa), α' (72 kDa) and β (53 kDa) (Thanh and Shibasaki 1977). The 11S globulin is composed of an acidic (38 kDa) and a basic polypeptide (20 kDa) linked by a single disulfide

bridge (Staswick and others 1981). Thus, the isoelectric points (pI) of 11S globulin is 6.4 which is higher than the pI of 7S globulin at 4.8 (Iwabuchi and Yamauchi 1987).

2.1.3 Health Benefits of Soy Protein

Soy foods have been staple diets for hundreds of years in Asian countries. Epidemiological investigations report that consumption of soy protein-foods could offer several health benefits. Human studies have shown that consumption of soy protein restrains risk factors for cardiovascular disease by lowering liver or blood triglyceride concentration, total and LDL cholesterol levels, increasing HDL cholesterol level and the ratio of HDL/LDL cholesterol (Anderson and others 1995). Consumption of at least 25g of soy protein per day could lower total and LDL cholesterol levels. The US Food and Drug Administration (FDA) approved a health claim for soy protein as lowering the risk of coronary heart disease in 1999. FDA requires any qualifying product to furnish at least 6.25 g of soy protein per serving, 25% of the necessary daily amount (25 g), with the expectation that foods containing soy protein would be eaten at least 4 times per day (Food and Drug Administration 1999).

Based on previous studies, it has been proposed that soy protein could lower the risk of diabetes complications including cardiovascular disease, nerve damage, and kidney damage (Clarkson 2002; Valsecchi and others 2008; Tovar and others 2002). Some animal and human studies indicated that soy protein could lower the postprandial glucose response and enhance insulin sensitivity. Some studies showed that soy protein was more effective than other types of proteins such as cod and milk protein (Lee 2006; Lavigne and others 2000; von Post-Skagegård and others 2006), whereas other studies showed no increased effectiveness (Liu and others 2010; Gobert and others 2010). These contrasting results could be attributable to variables such as

differences in the amount and source of protein, the presence of other ingredients that might affect glycemic control or diabetes complication risk factors, and difference in diabetic status among individuals in the studies. Nonetheless, since soy protein could lower total and LDL cholesterol it could overall reduce the risk of diabetes complications like heart disease.

2.1.4 Applications and Market Trend

In Asian countries, soy is often prepared in a diverse variety such as tofu (soybean curd), miso (fermented soybean paste), natto (fermented soybeans covered with mucilaginous substance), aburaage (fried sheet of tofu), and etc. Soy has long been a significant source of protein in the Asian diet (Nishinari 1988). Tofu-like foods often hold a fibrously firm texture that associates them with meat analogs to a certain extent. Recently, Chen and others (2009) further explained the formation of another soy-based product called yuba that is made from a film forming on top of heated soy milk which contains oil, particulate protein, soluble protein, and carbohydrate.

From 1992 to 2008, soy foods sales have experienced a substantial growth from US\$ 300 million to almost US\$ 4 billion. Factors leading to this growth may include the introduction of innovative soy products, the broader promotion of soy in the market, and the increasing number of vegetarians choosing soy-based foods for health and moral reasons (Soyfoods 2009). In addition to existing soy products in the market, soy beverage consumption is increasingly evident as it is considered an alternative over cow milk with even more potential health benefits. In the United States, the marketing cost for beverages formulated with soy has doubled since 2000 and the accumulating annual sales have exceeded US\$ 100 million (Beverage Marketing Corp. of New York 2005). The rapid market expansion both domestically and globally for soy beverage could lead to even larger market share for soy-based beverage in the near future.

2.2 Alginate and Its Health Benefits

2.2.1 Alginate

Alginate is both a biopolymer and a polyelectrolyte that is considered to be biocompatible, non-toxic, non-immunogenic and biodegradable (Klöck and others 1997; Mi and others 2002). It is an anionic polysaccharide linear in structure, with composition being two kinds of 1,4-linked hexuronic acid residues called β -d-mannuronopyranosyl (M) and α -l-guluronopyranosyl (G) residues. The arrangement of hexuronic acid residues is in repeating patterns, that is, blocks of repeating M residues (MM blocks), blocks of repeating G residues (GG blocks), and blocks of mixed M and G residues (MG blocks) (Matsumoto and others 1991). Alginate is widely found in the cell walls of brown algae. Its color ranges from white to yellowish. When dissolved in water, it can form a viscous gum. Its functionalities make it an excellent stabilizer and thickener when added to food products. Moreover, being an indigestible polysaccharide, alginate is considered a source of dietary fiber.

2.2.2 Health Benefits of Alginate

Dietary fibers including alginate are known for their health benefits. These include decreasing constipation and improving intestinal peristalsis, decreasing plasma lipid levels (Sola and others 2007), lowering blood pressure (Streppel and others 2005), and blood glucose control effects (de Leeuw and others 2004). Among these benefits, controlling blood glucose concentration is one of the most convincing effects. A recent review showed that the beneficial effects of high fiber foods may be best achieved in the context of a diet composed of foods with low glycemic index (Kendall and others 2010). In other words, increasing dietary fiber intake should be an effective approach to reduce postprandial glycemia and enhance insulin sensitivity (Paquet and others

2014). Alginate was also found to be of benefit to the diabetic population because of its potential effect in controlling blood glucose concentration. A number of cohort studies further illustrated the inhibition of risk of developing type 2 diabetes from increasing total dietary fiber intake (Schulze and others 2004). Moreover, a meta-analysis has been able to employ evidence from intervention studies on participants with type 2 diabetes to verify the active role of dietary fiber in improving glycemic control (Anderson and others 2004). Yet, the magnitude of effect that dietary fiber exerts on the glycemic response is dependable on its capacity to develop viscosity and gel during digestion (Juvonen and others 2009). Previous studies showed that dairy proteins (whey and casein) and negatively charged polysaccharide mixtures formed intragastric gelation when the mixtures were added to SGF (Zhang and others 2014c; Borreani and others 2016). Diffusion of food as well as the release of sugar was subsequently inhibited as intragastric gel entrapped them. In other words, they significantly reduced the glycemic response. Subsequently, this mechanism could help develop new insights in prevention and management of type 2 diabetes as delayed glucose absorption slows the blood glucose response, which is a major concern in type 2 diabetes. Furthermore, reducing dietary glycemic response has been proposed as a mean to combat the risk of diabetes and coronary heart disease (Livesey and others 2008).

2.3 Protein and Soluble Fiber Interactions

Protein ingredients and soluble fibers are often used together in many foods. Their unique gelling, emulsification and interfacial properties enable them to add textural and structural characteristics to food colloidal systems. The nature of the biopolymer and the environmental conditions determine the electrostatic-driven interactions between protein and anionic fibers in a different manner as shown in Figure 1: they can be either attractive or repulsive. Specifically, when the biopolymers repel with each other (incompatibility at $\text{pH} > \text{pI}$), the electrostatic

repulsion between protein and anionic soluble fiber can cause segregative phase separation. In contrast, the electrostatic interaction becomes associative when the biopolymers attract one another at $\text{pH} < \text{pI}$ (Tolstoguzov 1998). At high biopolymer concentrations, the extensive association can induce an unstable system and also cause phase separation (associative phase separation).

Figure 1. Protein and polysaccharide interactions in the mixture. Adapted from “Polysaccharide Protein Interactions” (De Kruif and Tuinier 2001).

Factors such as pH, ionic strength, temperature, biopolymer ratio and concentration also affect the type and degree of interaction between protein and soluble fibers. Among these factors, pH is the most significant factor that affects protein and soluble fiber due to its effect of charge density of the biopolymers. Controlling protein and soluble fiber interactions can be advantageous in designing food and beverage products with desirable structure and properties. Improvement of functional properties of commercial products has become the primary focus of research on interactions between soy protein and soluble anionic fibers (Xie and Hettiarachchy 1997; Martinez and others 2007).

The majority of studies on associative interaction focuses on interaction at $\text{pH} < \text{pI}$. However, at $\text{pH} > \text{pI}$, though the two biopolymers repel each other (segregation), attractive interaction can still occur between positively charged patches on the proteins and negatively charged fibers. (Dickinson 1998) reported the formation of soluble complexes between milk protein and polysaccharide at near neutral or even alkaline pH. The formation of complex consisting of β -LG and low- and high-methylated pectin at pH 7.0 was reported by (Girard and others 2002). Electrostatic complex between β -LG and dextran sulfate at near neutral pH showed improved heat stability (Vardhanabhuti and others 2009). Furthermore, a number of polysaccharides, namely carboxymethylcellulose (Huan and others 2016b) and λ -Carrageenan (Wang and others 2015), were noted in several reports for their interactions with whey protein when $\text{pH} > \text{pI}$.

2.4 Digestion Behavior of Mixed Protein/Soluble Fiber Systems

Protein digestion pattern is heavily influenced by several factors, namely, gastric conditions (pH, enzyme activity, and physiological surfactants), protein structure, and the presence of other food components in the gastrointestinal (GI) tract (Zhang and Vardhanabhuti 2014c; Zhang and others

2014b). There has been a notable rise of interest in understanding food digestion patterns in the GI tract as food related health issues continue to grow. Regarding digestive properties of protein, there have been studies showing that the rates and patterns of proteolysis can change significantly as a result of excessive food processing that changes protein structure (Hubbard 1998; Parsell and Sauer 1989). Recently, we have shown that the addition of anionic fibers such as pectin (Zhang and Vardhanabhuti 2014d), xanthan gum, and carrageenan (Zhang and others 2014b) could form an intragastric gel with protein when mixing with SGF, which could delay the degradation of foods (Hoad and others 2004; Kristensen and Jensen 2011; Hu and others 2017; Zhang and Vardhanabhuti 2014d). Implications of this mechanism might include slowing down gastric emptying, inducing satiety and lowered sucrose release profiles. In contrast to regular liquid food, the transformation of protein-containing liquid (e.g., beverages) into intragastric gel would require a longer transit time in the stomach.

The food industry has utilized the interactions between protein and fiber to improve stability, texture, and quality of food products (Aguilera 2005). Their interactions during digestion have been shown to influence the digestion pattern and delivery of nutrients *in vitro*. The formation of intragastric gel could be applied in designing foods to have improved glycemic control. It is important to determine the effect of intragastric gel on sucrose release and whether the results from *in-vitro* will be confirmed in the human study.

CHAPTER 3

MANUSCRIPT 1: INTRAGASTRIC GELATION OF HEATED SOY PROTEIN ISOLATE/ALGINATE MIXTURE AND ITS EFFECT ON SUCROSE RELEASE

Manuscript to be submitted to Journal of Food Science

3.1 Introduction

Soy is one of the most nutrient-rich legumes due to its high protein and well-balanced amino acid composition. Soy protein has been widely regarded as a key ingredient in food formulation to enhance nutrients, functionalities, and qualities. Soy protein-containing foods potentially possess a number of health benefits, namely lowering the risk of heart disease and diabetes (Erdman 2000; Anderson and others 1995; Friedman and Brandon 2001). With consumers' awareness of these benefits and the increasing popularity of protein-based drinks, the market of soy protein-containing foods, especially soy protein beverages, has been experiencing a sizable growth (Granato and others 2010). Two main fractions of soy protein, 7S globulin (β -Conglycinin) and 11S globulin (glycinin), represent more than 85% of the storage proteins in the seed. The 7S globulin is a heterogeneous glycosylated trimeric glycoprotein consisting of at least six combinations of three subunits: α (76 kDa), α' (72 kDa) and β (53 kDa) (Thanh and Shibasaki 1977). The 11S globulin is composed of an acidic (38 kDa) and a basic polypeptides (20 kDa) linked by a single disulfide bridge (Staswick and others 1981).

Understanding factors affecting digestion properties of food systems could lead to the design of food products with improved health benefits. The pattern of protein digestion in the gastrointestinal (GI) tract is highly dependable on several factors such as gastric conditions,

physical properties of the protein, and the presence of other food components in the matrix. Various kinds of polysaccharides such as pectin, cellulose, and alginate are often used in protein-based products to modify the viscosity as well as improve the texture and stability of food or beverages. The effects of soy protein and polysaccharide interactions on functional properties have been reported (Yuan and others 2013; Lam and others 2007; Lam and others 2008; Jaramillo and others 2011); however, whether and how polysaccharides influence soy protein digestion is not fully understood. Previous studies showed that solutions of mixed dairy proteins (whey and casein) or soy protein and negatively charged polysaccharide formed intragastric gelation when the mixtures were added to simulated gastric fluid (SGF) (Zhang and others 2014c; Borreani and others 2016; Hu and others 2017). Intragastric gelation is believed to derive from the electrostatic interactions between positively charged patches on the proteins and negatively charged polysaccharides when pH changes from near neutral to acidic under simulated gastric conditions. Protein digestion significantly decelerates when intragastric gel is formed. Implications of this mechanism might include slowing down gastric emptying and inducing satiety.

Another potential effect of intragastric gel formation is its impact on the release of nutrients and food ingredients such as sugar. In contrast to regular liquids, the transformation of a soy protein-containing liquid (e.g., beverages) into intragastric gels would require longer transit times in the stomach. Thus, it is highly possible that the nutrients entrapped inside the intragastric gel would need longer time to reach the small intestine (Hur and others 2011). Soluble dietary fibers have been shown to effectively regulate the postprandial glucose levels by altering food texture, structure, and viscosity (Brennan 2005). Bakalis and others (2007) used a human intestine model to demonstrate this mechanism: they employed guar gum solution to increase food viscosity so

that flow of material to the GI tract would slow down, which would eventually exert an impact on digestion and uptake of other nutrients. Along with intragastric gelation, it is believed that food viscosity also plays a role in the digestion pattern of protein in the fiber-enriched beverages as well as satiety.

Reducing the dietary glycemic response has been proposed as a means to combat the risk of diabetes and coronary heart disease (Livesey and others 2008). A recent review showed that the beneficial effects of high fiber foods may be best achieved in the context of a diet composed of foods with low glycemic index (Kendall and others 2010). In other words, increasing dietary fiber intake is a convincing approach to reduce postprandial glycemia and enhance insulin sensitivity (Paquet and others 2014). Yet, the magnitude of effect that dietary fiber exerts on the glycemic response depends on its capacity to develop viscosity (Juvonen and others 2009).

Nonetheless, ingestion of fiber could slow down the rate of gastric emptying and decrease the absorption of glucose in the lumen of the small intestine. As previously stated, the fiber-enriched protein solutions formed a gel by self-structuring once it entered a gastric environment (Zhang and others 2014c; Hu and others 2017; Zhang and others 2014b). Formation of intragastric gel may delay the glycemic response even further similar to the delayed absorption of solid foods (O'Dea and others 1980; Collier and O'Dea 1982; Björck and others 1994).

The objective of this study was to investigate the effect of alginate, an anionic fiber, on digestion properties of soy protein isolate (SPI) in model beverages. The effect of alginate concentration and pH were studied. Digestion pattern of protein as well as the sucrose release profile were characterized.

3.2 Materials and Methods

3.2.1 Materials

SPI (Pro-Fam[®] 921, Archer Daniels Midland Company, Decatur, IL) contained 90% protein, 4% fat and 5% ash as stated by the manufacturer. Alginate was provided by Danisco USA Inc. (New Century, KS). Sucrose was purchased from Fisher Scientific (Fair Lawn, New Jersey). Pepsin from porcine gastric mucosa was obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all of the chemicals used were of analytical grade.

3.2.2 Heat treatment of SPI/alginate Mixtures

Stock solutions of SPI (10% w/w), alginate (1% w/w), and sucrose (50% w/w) were prepared by dissolving powders in Millipore water (17.0 MΩ) with continuous stirring for at least 2 h at ambient temperature. The stock solutions were then kept in the refrigerator (4 °C) overnight for complete hydration. Stock solutions of SPI and alginate were mixed with sucrose and water at the appropriate amount. The pH of the mixed solutions was adjusted to 6.0 and 7.0 using 0.1N and 0.01 N HCl respectively. Water was added such that the final solutions contained 5% w/v protein, 10% w/v sucrose and 0-0.2% w/v alginate. The mixtures were gently mixed before being heated in a temperature-controlled water bath at 85 °C for 30 min and then cooled using running tap water.

3.2.3 Zeta-potential measurements

The average electrical charge (zeta-potential) of model beverages was measured by dynamic light scattering using the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) at 25 °C. Samples were diluted 20 times using 5 mM sodium phosphate buffer solutions adjusted to the sample pH before measurement. Three replications were tested for each sample.

3.2.4 Particle size measurements

The particle size of model beverages was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. During the measurement, the laser light was directed and focused on the cuvette with sample solutions. Before testing, the model beverages were diluted 20 times with 5 mM sodium phosphate buffer solutions having the same pH as the beverages. For each sample, three measurements were conducted with at least 12 runs and each run lasted for 10 s. The relative refractive index was set as 1.06 for the SPI particle size measurements as mentioned in the former study (Lam and others 2007). The whole experiment was replicated three times.

3.2.5 Rheological properties

Shear stresses developed with applied shear rates ranging from 1 to 100 s⁻¹ for model beverages were measured with a Kinexus rheometer (Malvern Instruments Ltd., Worcestershire, UK). All tests were performed at room temperature (25°C) using a cone-plate geometry (40 mm) and a constant gap of 0.05 mm. These flow curves were modeled using the Power Law model:

$$\tau = k \cdot \gamma^n$$

Where τ is shear stress, k is consistency coefficient, γ is shear rate, and n is flow behavior index. Apparent viscosity values between different samples were compared at a shear rate of 50 s⁻¹. Measurements were performed in duplicate.

3.2.6 Digestion experiments

Bio-Dis reciprocating cylinder apparatus 3 (Agilent Technologies, Santa Clara, CA) was used in digestion experiments according to Pharmacopoeia official methods (Zhang and others 2014c).

The temperature of the digestion media was maintained at 37 ± 0.5 °C using a digitally controlled water circulation and heater system. Simulated gastric fluid (SGF) was based on the work by Minekus and others (2014) with modifications. Pepsin solution was prepared freshly before adding into SGF, the composition is shown in Table 1. Model beverage (25 mL) was added into 75 mL of digestion media such that the amount of pepsin was at 2000 U/mL in the final digestion mixtures. The pH of the mixtures was readjusted to 3.0 using 1-2 drops of 1N and 0.1N NaOH or HCl solutions if necessary. The mixtures were then gently transferred into the internal cylinder. The digestion experiments were performed at a reciprocating rate of 20 dips per minute (dpm) using 405 µm mesh screens. If intragastric gel was formed, the gel remained in the internal cylinder while the liquid freely flowed into the external cylinder. This process was completed with the system as shown in Figure 2 (Pezzini and others 2015). Samples (2 mL) for electrophoresis and HPLC analysis were taken manually from the external cylinder at time intervals of 5, 10, 20, 30, 60 and 120 min and replenished with 2 mL fresh digestion media. NaOH (1 N and 0.1 N) was added to the samples to adjust their pH to 7.5 in order to inactivate the enzyme. The total volume of the samples was adjusted to 4 mL by adding DI water. For samples at time 0, model beverages were mixed with SGF without pepsin addition.

Table 1. Final concentrations of constituents before addition to the sample of the simulated gastric fluid (SGF).

SGF	
constituent	pH 3
mmol L⁻¹	
KCl	5.52
KH ₂ PO ₄	0.72
NaHCO ₃	20.00
NaCl	37.76
MgCl ₂ (H ₂ O) ₆	0.08
(NH ₄) ₂ CO ₃	0.40
HCl	32.48
CaCl ₂ (H ₂ O) ₂	0.12
U mL⁻¹	
pepsin	2667

Figure 2. Reciprocating cylinder apparatus 3: (a) Internal cylinder and its top and bottom caps (b) Internal cylinder coupled to the rod inside the external cylinder. Adapted from “Applications of USP apparatus 3 in assessing the in vitro release of solid oral dosage forms” (Pezzini and others 2015)

3.2.7 Electrophoresis

A modified Laemmli method previously used by our lab (Zhang and others 2014c) was employed for SDS-PAGE analysis. Samples taken during the digestion experiment were solubilized in a Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) that contained 5% β -mercaptoethanol with the ratio of original sample to Laemmli sample buffer of 1:1. Once the samples were solubilized, they were heated at 95 °C for 5 min, cooled to room temperature, and loaded (20 μ L) onto the 4-20% acrylamide precast gel (Bio-Rad Laboratories, Hercules, CA). The electrophoresis was conducted in a Mini-Protean Tetra electrophoresis system (Bio-Rad Laboratories, Hercules, CA) using an electrode stock buffer at a voltage of 100 V. The gels were marked with Coomassie Brilliant Blue R250 staining solution consisting of acetic acid, methanol, and H₂O with a 1:4:5 ratio in volume, and destained in an acetic acid:methanol:H₂O solution (1:4:5 by volume). A pre-stained molecular weight marker comprising a range of protein with sizes that varied from 10 to 250 kDa was used (Precision Plus Protein™ Dual Xtra Prestained Protein Standards, Bio-Rad Laboratories, Hercules, CA). Gels were documented using a Gel Doc EZ Imaging System (Bio-Rad Laboratories, Hercules, CA).

3.2.8 Sucrose release profile

Samples that were taken out during the digestion experiment were filtered through a 0.45 μ m filter (PVDF, Millipore Corporation, Bedford, MA) before HPLC measurement. HPLC analysis was performed on a Bio-Rad Aminex HPX-87H ion exclusion column attached to a Perkin Elmer LC pump system (Series 410, Waltham, Massachusetts). The mobile phase contained 0.045 N H₂SO₄ and 6% acetonitrile in HPLC grade water. Sample injection volume was 20 μ L, the flow rate was 0.5 mL/min, and separation was performed at 55 °C column temperature for 30

min. The RI detector (RID-6A, Shimadzu) was used as a concentration detector. The analysis of sucrose was performed in duplicate batches. Duplicate determinations of standard samples at six different concentrations were established for the calibration curve.

3.2.9 Statistical analysis

A one-way analysis of variance (ANOVA) was performed using the SAS version 9.3 software (SAS Institute, Cary, NC) to determine the statistical difference. Significant differences ($P < 0.05$) between different samples were determined by Tukey's Studentized Range test (HSD).

3.3 Results and Discussion

Heating is the common processing step in beverage manufacturing. Varying biopolymer concentrations and pH during heating can influence the properties of the mixed protein and polysaccharide systems which in turn will influence their functional properties such as heat stability as well as their digestion properties. In this study, the particle size and electrical charge of the soluble aggregates formed from heating mixed SPI and alginate were characterized.

3.3.1 Zeta-potential (ζ -potential)

Zeta potential measurement was used to determine the surface charge properties of the soluble aggregates. The ζ -potentials of model beverages without alginate were -23.5 ± 0.71 and -26.1 ± 1.02 mV at pH 6.0 and pH 7.0, respectively (Figure 3), as expected based on the negative charges of the protein above its isoelectric point (pI). Heated SPI at pH 7.0 showed lower ζ -potential than at pH 6.0 due to the release of bound protons from various functional groups. The ζ -potential of alginate was -45.7 ± 2.0 and -46.3 ± 1.9 mV at pH 6.0 and 7.0, respectively. Addition of alginate resulted in decreasing zeta-potential for both pH 6.0 and pH 7.0 samples. Previous

work on the effect of pectin on SPI showed that pectin formed complexes with SPI at a pH below or near pI as shown by a decrease in ζ -potential. However, at pH 6 and 7, the ζ -potentials were not affected suggesting any complex formation (Jaramillo and others 2011). In this study, as alginate concentration increased the ζ -potentials decreased, suggesting a certain degree of SPI-alginate interactions even at pH > pI. Similar results were reported with whey protein isolate and pectins (Zhang and others 2014a; Zhang and Vardhanabhuti 2014a), whey protein isolate and carrageenan (Wang and others 2015), as well as whey protein isolate and carboxymethylcellulose (Huan and others 2016a). When the pH is above pI both biopolymers carry net negative charge and the repulsive force between the molecules could drive the system into a segregated system. However, some positively charged patches remain present on the protein and they could interact and form soluble complexes with the polysaccharide (Dickinson 1998). The interactions could be favored during heating (Zhang and others 2014a). Regression analysis showed a linear relationship between ζ -potential (y) and alginate concentration (x) for both pH 6 and 7 (Eqs. 1 and 2, respectively). The larger decrease in ζ -potential at pH 6 further supported that electrostatic interaction could occur at these pH values. SPI molecules could have a higher number of positively charged sites at pH 6.0 than pH 7.0, thus more alginate molecules can bind to the exposed cationic amino acid groups.

$$\text{At pH 6,} \quad y = -30.733x - 23.24, R^2 = 0.9746 \quad \text{Eq. 1}$$

$$\text{At pH 7,} \quad y = -19.6x - 26.307, R^2 = 0.9869 \quad \text{Eq. 2}$$

Figure 3. Zeta potential of model beverages containing 5% (w/w) protein, 10% (w/w) sucrose and 0 to 0.20% (w/w) alginate at pH 6.0 (●) and pH 7.0 (■) after heating treatment

3.3.2 Particle size

Particle size is one of the most important parameters to determine the stability of the colloidal system. The particle size properties of model beverages are shown in Figure 4. Without the addition of alginate, the particle size of model beverages was similar at pH 6.0 than pH 7.0.

At both pH 6.0 and 7.0, the sizes of the soluble aggregates increased with increasing alginate concentration after the beverages were heated. This phenomenon could be attributed to the fact that protein molecules carry high negative charges at both pH, and, even though some alginate can interact with positively charged groups (as shown above), the overall repulsive interaction condition could drive the mixtures towards segregative phase separation. As a result, larger protein aggregates with increasing alginate concentration were observed. Similar results were reported that the particle size of heated whey protein and pectin complex increased with increasing pectin content at $\text{pH} > \text{pI}$ (Zhang and Vardhanabhuti 2014b; Zhang and others 2012). However, no difference in particle size was found in heated SPI and pectin system at pH 6.0-7.0 (Jaramillo and others 2011). Differences in the results were probably due to different type and concentration of polysaccharide (much higher alginate concentration in our study). The model beverages prepared at pH 6.0 showed larger aggregate sizes than at pH 7.0 when the alginate concentration was higher than 0.15% ($p < 0.05$). This outcome signified varying degrees of interactions that occurred. At high alginate concentrations, the segregative phenomenon is supposed to be stronger at pH 7 since proteins are more negatively charged. However, at pH 6 protein-protein interactions are favored. Formation of larger aggregates at pH 6.0 indicates that higher degree of protein-protein interactions (at pH 6) led to more extensive aggregation compared the segregative effect at pH 7. It should be noted that no observation phase separation or precipitation was observed in any of the samples even after 24 h under refrigeration.

Figure 4. Z-average diameter of model beverages prepared with 0-0.20% alginate at pH 6.0 (●) and pH 7.0 (■) after heating treatment.

3.3.3 Rheological properties

Viscosity is an important property determining the quality and acceptability of beverages. It is also highly correlated with digestion property. Both *in vitro* (Sasaki and Kohyama 2012) and *in vivo* studies (Zijlstra and others 2012) reported that polysaccharides (eg. xanthan gum, guar gum, konjac glucomannan, and pectin) can affect the viscosity and digestibility of food. As shown in Table 2, all heated model beverages exhibited pseudoplastic behavior, which was initiated by the application of shear flow that broke the molecular entanglements. Along the flow field, Brownian motion was overcome due to the more organized molecules that offered less resistance. As a result, viscosity decreased as the shear rate increased (Sun and others 2007; McClements 1999). Interactions between protein and polysaccharide is one major factor influencing the rheological properties. Specifically, stronger electrostatic interactions between protein and polysaccharide could lead to changes in viscosity and flow behavior (Benichou and others 2002; De Kruif and others 2004). In this study, the model beverages at both pH values exhibited more obvious pseudoplastic behavior as shown by lower flow behavior index (n) and higher viscosity as shown by higher consistency coefficient (k) with added alginate. The apparent viscosity at 50 s^{-1} (swallowing shear rate) also increased with increasing alginate concentration. These changes in rheological behaviors revealed a higher level of electrostatic interactions between the biopolymers. At 50 s^{-1} , model beverages containing 0.2% alginate showed approximately 3.4-fold and 2.8-fold increase in viscosity compared with SPI without alginate at pH 6.0 and 7.0, respectively. The effect of pH was less apparent. It should be noted that the highest viscosity (0.0248 Pa.s) observed in this study was still much lower than the normal commercialized protein shakes reported as around 0.150 Pa.s (National Dysphagia Diet

Task Force and American Diabetic Association 2002). Thus, all samples should be acceptable to consumers.

Table 2. Rheological properties of model beverages prepared with 0-0.20% alginate at pH 6 and 7 after heating treatment.

pH	Alginate (%)	n*	k (Pa·s ⁿ)*	Viscosity at 50 s ⁻¹ (Pa·s)
6.0	0	0.831 ± 0.026 ^{bc#}	0.0131 ± 0.0014 ^{ab}	0.0055 ± 0.0001 ^a
	0.05	0.846 ± 0.025 ^c	0.0144 ± 0.0012 ^{ab}	0.0073 ± 0.0003 ^{ab}
	0.10	0.750 ± 0.047 ^a	0.0280 ± 0.0034 ^{cd}	0.0102 ± 0.0005 ^{bcd}
	0.15	0.775 ± 0.014 ^{ab}	0.0351 ± 0.0009 ^d	0.0146 ± 0.0008 ^e
	0.20	0.713 ± 0.014 ^a	0.0556 ± 0.0073 ^e	0.0185 ± 0.0013 ^f
7.0	0	0.919 ± 0.016 ^d	0.0127 ± 0.0014 ^a	0.0089 ± 0.0006 ^{bc}
	0.05	0.835 ± 0.010 ^{bc}	0.0237 ± 0.0016 ^{abc}	0.0117 ± 0.0015 ^{cd}
	0.10	0.853 ± 0.010 ^{cd}	0.0240 ± 0.0007 ^{bcd}	0.0129 ± 0.0005 ^{de}
	0.15	0.862 ± 0.023 ^{cd}	0.0256 ± 0.0036 ^{cd}	0.0150 ± 0.0016 ^e
	0.20	0.833 ± 0.022 ^{bc}	0.0470 ± 0.0079 ^e	0.0248 ± 0.0015 ^g

* n and k are the powder law model parameters: flow behavior index and consistency coefficient.

Each value is an average of three samples ± standard deviation. Means within a column not sharing a letter are significantly different (P<0.05, Tukey's test).

3.3.4 Intragastric gelation

Figure 5 represents the behavior of heated samples once they were mixed with SGF (time 0).

The ability of the mixtures to form intragastric gel depends on both the concentration of alginate and the pH of model beverages. With the exception of SPI without alginate at pH 7.0, all beverages samples immediately formed intragastric gel when mixed with SGF. SPI beverage at pH 6.0 formed weak intragastric gel even without alginate. Gels appeared to be more well-defined at higher alginate concentration and in beverages heated at pH 6 compared to pH 7. It should be noted that alginate alone at 0.2% (w/w) did not form gel when mixed with SGF.

Without alginate, the mechanism of intragastric gelation of the model beverages at pH 6 could be protein aggregation due to the abrupt change in pH once it was mixed with SGF. In the absence of alginate, pH 7.0 sample did not form intragastric gel since protein molecules have limited interactions with each other during the pH change. In the presence of alginate, intragastric gelation was enhanced due to the electrostatic interactions between carboxylic groups of alginate and the amino groups of soy protein. Negatively charged alginate had limited interaction with protein during heating around neutral pH. However, when mixed with SGF (e.g., the final pH of the mixture was 3.0), SPI became positively charged and could bind with negatively charged alginate. The attractions between SPI and alginate molecules were positively proportional to the alginate concentrations. This is due to the increasing number of alginates available to associate with protein molecules to form an even stronger cross-linked gel network. Similar results were reported during in-vitro gastric digestion of mixed whey protein and pectin (Zhang and Vardhanabhuti 2014d) as well as gelation of SPI and xanthan gum or carrageenan in GI tract (Hu and others 2017).

(a)

(b)

Figure 5. Intragastic gel or fluid of the model beverages prepared with no alginate (A and D), 0.10% alginate (B and E), and 0.20% alginate (C and F) at pH 6.0 (a) and pH 7.0 (b) after mixing with SGF.

3.3.5 Electrophoresis

SDS-PAGE was used to determine the *in vitro* digestion patterns of SPI in the six model beverages prepared with different alginate concentration and pH. Figure 6 shows the digestion profiles of protein after digestion for 0 to 2 h. SPI subunits of 7S protein fraction (α , α' and β subunits) and 11S protein fraction (subunits of A and B) were labeled. The figures showed that the digestion pattern of protein was affected by alginate concentration and pH of the model beverages.

As shown in Figure 6A, SPI sample heated at pH 7.0 with no alginate showed clear bands of all major soy protein fractions at time 0 (lane 4). These bands were similar to those from unheated SPI control (lane 3). Within 5 min of digestion, the majority of monomer bands disappeared while there appeared bands with MW less than 15 kDa, indicating that most protein fractions were digested into peptides. Increasing digestion time led to more digested proteins as shown by increased intensity of the peptide bands. The decrease in band intensity at the end of digestion indicated that proteins were further digested into smaller peptides and/or amino acids. For SPI beverage without alginate at pH 6.0, only light bands were shown at time 0 (lane 4, Figure 6B). This indicates that most of the protein molecules were present in the intragastric gel and were not detected in the aqueous phase (external tube). After 5 min, proteins in the gel started to be digested as shown by the appearance of monomer bands. As digestion progressed, peptide bands were more intense, and, similar to sample at pH 7, peptide bands started to fade at the end of digestion. When alginate was added to the beverages at both pH no monomer band was shown at time 0 due to the formation of intragastric gel. Protein was slowly digested as digestion progressed. The digestion rate decreased at a higher alginate concentration as shown by weaker bands (Figure 6E and 6F). Model beverages prepared at pH 6.0 showed slower digestion rate

than pH 7.0 as shown by the less intense bands throughout digestion. It should be noted that at 0.2% alginate intragastric gel still remained at the end of digestion with samples at pH 6 showing higher amount of undigested gel (data not shown). This result corresponds to the observation that the intragastric gels formed from beverages at pH 6 were more well-defined and more resistant to digestion.

Previous studies have shown that negatively charged polysaccharides could significantly slow the digestion rate of protein by the formation of intragastric gel (Zhang and others 2014b). Digestibility of the intragastric gel depended on the strength of the gel which was the result of the degree of association between protein and polysaccharides. In this study, increased alginate concentration led to higher number of interactions between the two biopolymers. At pH 6, the protein is less negatively charged compared to at pH 7, thus electrostatic association between the positively charged patches on the protein and the negatively charged alginate could be more favorable during heating. These enhanced interactions could promote extra attractive interconnections between protein and alginate gel network when the pH was lowered under simulated gastric conditions. In addition, enhanced interactions could also mean fewer accessible sites for pepsin, leading to a slower digestion rate of the protein.

Figure 6. SDS-PAGE of protein during in-vitro digestion of model beverages prepared with no alginate (A and B), 0.10% alginate (C and D), and 0.20% alginate (E and F) at pH 6.0 and pH 7.0: Lane 1, standard marker; lane 2, pepsin; lane 3, unheated SPI; lanes 4–10, beverage samples digested for 0, 5, 10, 20, 30, 60, and 120 min, respectively.

3.3.6 Sucrose release profile

Figure 7 shows the sucrose release profiles of beverages during the *in vitro* gastric digestion as determined by HPLC. The data at 120 min are not shown since all sucrose was completely released after 30 min digestion in all samples. Model beverage with no alginate at pH 7.0 dissolved in SGF immediately without forming intragastric gelation, thus the sucrose content was constant from the beginning until the end of the digestion. However, for the beverage without alginate at pH 6.0, this sample formed a weak and less well-defined intragastric gel which trapped about 12.1% sucrose. Note that the electrophoresis results showed that most of the protein was not detected (lane 4, Figure 6B). It is likely that the weak protein network could not trap sucrose; however, the gel/aggregates (e.g., consisted mostly of protein) were larger than 405 nm and could not pass through the mesh screen of the internal cylinder. When beverages contained 0.1% alginate sucrose release at time 0 decreased to 40.5% and 35.8% for samples at pH 7.0 and 6.0, respectively. At 0.2% alginate, even less sucrose was detected at time 0 and a lower amount of sucrose was released during digestion compared to samples at 0.1% alginate. At similar alginate concentrations, beverages at pH 6.0 showed a lower amount of sucrose release from time 0 to 30 min compared to those at pH 7.0. Thus, overall a lower amount of sucrose at time 0 and slower sucrose release rate were exhibited in samples with high alginate concentration and at pH 6.0. These results indicated that the formation of well-defined intragastric gel immediately trapped sucrose in the gel network. As digestion progressed, pepsin and the mechanical action broke down the gel network and more sucrose was released. Even though the gels could not be fully digested within 30 min, sucrose was completely released in this period, indicating that sucrose diffused from the gel matrix as gel was immersed in the gastric fluid. The alginate concentration appeared to be more pronounced compared to the pH in

delaying the sucrose release from the matrix. These results suggest that protein beverages could potentially be formulated to form intragastric gel and to have lower postprandial glucose release property. We are currently conducting a clinical trial to prove this hypothesis.

Figure 7. *In vitro* sucrose release profiles of model beverages prepared with different alginate concentrations at pH 6.0 (a) and pH 7.0 (b): no alginate (●), 0.10% alginate (■), and 0.20% alginate (▲).

3.4 Summary

Heating SPI and alginate at $\text{pH} > \text{pI}$ resulted in the formation of soluble aggregates with larger size and more negatively charged. The size and charge of the aggregates depended on alginate concentration and pH. The addition of alginate also led to beverages with higher pseudoplastic behavior and higher viscosity. Under *in vitro* gastric digestion, digestibility of SPI highly depends on the alginate concentration and, to a lesser extent, pH. Model beverages prepared at pH 6.0 were more resistant to digestion and exhibited slower sucrose release than those at pH 7.0 at the same alginate concentration. Increased alginate concentration resulted intragastric gel that was more resistant to digestion and released sucrose at the slower rate. We are currently conducting clinical trial to determine whether consumption of beverages that formed intragastric gel and had slower sucrose release profile will lead to lowered and/or slower postprandial blood sugar response.

CHAPTER 4

MANUSCRIPT 2: EFFECTS OF MIXED SOY PROTEIN ISOLATE AND ALGINATE BEVERAGES ON POSTPRANDIAL GLUCOSE RESPONSE AND APPETITE IN HEALTHY ADULTS

Manuscript to be submitted to Journal of Nutrition

4.1 Introduction

According to WHO, the number of adults living with diabetes has almost quadrupled since 1980 to 422 million worldwide (World Health Organization 2016). This number will only continue to climb as the aging population, diabetes rate, and pre-diabetic rate increase. It is estimated that the diabetes-related cost in the United States alone was \$245 billion in 2012 (Centers for Disease Control and Prevention 2014). Furthermore, diabetics are exposed to a higher risk of developing diabetes-related complications and they are especially vulnerable to diseases such as microvascular and cardiovascular diseases (CVD). Previous studies reveal that, among prevention and treatment programs, improving glycemic control could be effective in reducing the risk of diabetes complications, especially for the newly diagnosed patients (Holman and others 2008; Turnbull and others 2009).

Both fasting glucose and postprandial plasma glucose concentration are directly correlated to the risk of diabetes complications, with postprandial concentration potentially constituting a stronger risk factor for cardiovascular complications (Levitan and others 2004). Dietary management can be an effective approach to control postprandial plasma glucose concentration. The glycemic index is a numerical value assigned to a particular type of food that denotes its magnitude of

effect on postprandial blood glucose responses (Jenkins and others 1981). It is defined as the area under the plasma glucose curve (AUC) for the tested food, expressed as a percentage of a standard control. Intake of foods with low glycemic index not only help lower the postprandial glucose response but also potentially sustains insulin secretion, which further improves insulin sensitivity and regulates blood glucose levels (Miller and others 1996). Additionally, absolute blood glucose area (absolute AUC) is a powerful parameter in blood glucose control that indicates fluctuation of blood glucose. Subsequently, the blood glucose control effect can be shown by reduction in the postprandial glucose response, as well as both incremental blood glucose area above the baseline (incremental AUC) and absolute AUC. With improved blood glucose control, the risk of complications associated with diabetes could be noticeably reduced (Nathan and others 2009; Wang and others 1993; Ray and others 2009).

Recently, there is increased interest on the effect of food components or ingredients on diabetes. It has been proposed that development of diabetes complications could be significantly inhibited with intake of soy protein. Upon entering the gastrointestinal (GI) tract, protein would start slowing gastric emptying, subsequently lowering the postprandial glucose concentration (Campbell and Rains 2015). Soy protein was found to be potent, even more effective than casein, in controlling blood glucose concentration in animals (Lee 2006; Lavigne and others 2000). Its effect has been confirmed in human studies (von Post-Skagegård and others 2006; Lang and others 1999). Moreover, soy protein consumption has been notable in reducing total and LDL cholesterol as well as the risk factors of heart disease. Thus, the health benefits of soy protein consumption could contribute to overall reduction in diabetes risk.

Dietary fiber can also potentially reduce the risk of diabetes and diabetes complications. Previous human studies have shown the positive effect of dietary fiber on reducing the incidence of

diabetes (Salmeron and others 1997; Schulze and others 2004; Meyer and others 2000; Montonen and others 2003) and diabetes complications including cardiovascular disease, nerve damage, and kidney damage (Clarkson 2002; Valsecchi and others 2008; Tovar and others 2002). Soluble dietary fibers have been shown to be effective in regulating the postprandial glucose levels by altering food texture, structure, and viscosity (Brennan 2005). Using a model intestine, Bakalis and others (2007) demonstrated that food modified to have increased viscosity by guar gum had significantly slower flow rate in the GI model. As a result, regulation in postprandial glucose levels was achieved as digestion and uptake of nutrients decelerated. A viscous bolus inhibited the bulk diffusion of food. Finally, postprandial glucose and insulin responses were significantly hindered (Fabek and others 2014; Kendall and others 2010). This mechanism for glycemic control could be applied in prevention and management of type 2 diabetes (Juntunen and others 2002).

Recent studies showed that solutions of mixed dairy proteins (whey and casein) and negatively charged polysaccharide formed intragastric gelation under *in vitro* gastric digestion (Zhang and others 2014c; Borreani and others 2016; Zhang and Vardhanabhuti 2014e). The gelation mechanism is proposed to be the crosslinking between oppositely charged protein and polysaccharide when pH is reduced to below the pI of the protein. In our previous study, model beverages formulated with soy protein and alginate could form intragastric gel once they are mixed with simulated gastric fluid (SGF), resulting in delayed protein digestion as well as slower sucrose release (Huang and Vardhanabhuti in preparation). The question is whether the result from *in vitro* study would be significant *in vivo*. If intragastric gelation happens *in vivo*, glycemic responses may be delayed due to the decelerating rate of absorption of glucose. Apart from

delaying glycemic response, the formation of intragastric gel might slow down gastric emptying and induce satiety at the same time.

This study investigated whether consumption of beverages containing soy protein and alginate, that show intragastric gelation in a model stomach, would influence the postprandial glucose response as well as appetite in healthy subjects. The outcome of this study could lead to reformulation of beverages and semi-solid food products to have improved glycemic control.

4.2 Materials and Methods

4.2.1 Subjects

A sample size of $n = 12$ (4 men and 8 women, age 23.3 ± 0.8 years old, BMI 20.9 ± 0.8 kg/m², fasting blood glucose 91.3 ± 1.8 mg/dL) was included in this study. Fifteen nondiabetic, healthy subjects were recruited at the University of Missouri-Columbia campus by means of online advertising and flyers distributed. Three participants were excluded because of changes of medication within 6 months before testing. Inclusion criteria were as follows: (1) 18-30 years old; (2) normal to overweight (BMI: 18–28 kg/m²); (3) no metabolic disease and fasting blood glucose level < 100 mg/dL; (4) not been clinically diagnosed with an eating disorder; (5) not currently/previously on a weight loss or other special diet (in the past 6 months); (6) not a smoker (in the past year) (7) habitually eat (i.e., at least 5 times/wk) breakfast between 7:00-9:00 am and lunch between 11:00 am-2:00 pm; (8) no food allergies or intolerances to soy and dairy products; (9) not taking any medications, or having had any changes in medication within the past 6 months, that could influence the study outcomes; (10) not pregnant; and (11) rating the palatability of the study treatments as greater than or equal to “neither like nor dislike”. The potential participants were excluded if they did not meet the inclusion criteria. To minimize the

variability, they were informed to fast overnight (>8 h) prior to the screening day. Study procedures were approved by the MU Institutional Review Board and all participants were provided with informed consent. The participants received \$180 for completing all study procedures.

4.2.2 Beverage Preparation

Formulations of 6 selected beverages are shown in Table 3. CONT was the control sample with 10% sugar and 3% vanilla flavor only. ALG contained sucrose and 0.25% alginate. The total amount of 0.625 g alginate was much lower than the concentrations reported in studies showing the effect of alginate on blood glucose response. Since our study aimed at determining the effect of mixed protein and fiber, we selected this low of a concentration of fiber so that the effect of alginate alone would not be significant. Protein treatments (SPI-6 and SPI-7) contained 5% protein at pH 6 and 7, respectively. Our previous work showed differences in digestion behavior of SPI at different pH, thus samples at two different pH were selected. Treatments containing both SPI (5%) and alginate (0.25%) were also prepared at pH 6 and 7 (SPI+ALG-6 and SPI+ALG-7, respectively).

All ingredients were commercially available and were GRAS (generally recognized as safe) listed. SPI and alginate were separately dissolved in drinking water before being mixed together in appropriate amounts. Sugar (25 g) and 7.5 g vanilla flavor were then added and the pH of the mixtures was adjusted accordingly. Drinking water was added such that the compositions of the samples were as listed in Table 3. The beverages were then heated at 85 °C for 30 min. The beverages were prepared in either the Food Science or MUNCH Facility in advance, labeled with the participant ID, and placed in the refrigerator in an airtight container until the following

testing day. The participants were required to consume all of the beverages given to them within 10 min.

Table 3. Dietary Characteristics of each beverage.

	Test Beverage					
	CONT	ALG	SPI-7	SPI-6	SPI+ALG -7	SPI+ALG -6
Energy Content (kcal)	122	122	172	172	172	172
Total Protein (g)	0	0	12.5	12.5	12.5	12.5
Alginate (g)	0	0.625	0	0	0.625	0.625
pH	7.0	7.0	7.0	6.0	7.0	6.0

*Values are estimated to the nearest gram. All samples contained 25 g sugar and 7.5 g vanilla flavor out of 250 g of beverage.

4.2.3 Study Design

The study was a randomized, double-blind, crossover study in which all participants completed each of the 6 testing days. The randomization scheme regarding the order of beverages given to each participant was created using the following Research Randomizer program: www.randomizer.org. The study required individual participants to complete the study in 6 testing days, each of which lasted 3 hours. A 1-7 day washout period occurred between treatments. On each testing day, participants reported to the University of Missouri-Physical Activity and Wellness Center (MU-PAW) after an overnight fast. Each participant was seated in a reclining chair and for the next 30 min, simply acclimated to the room and familiarized with the testing day procedures. After the participant felt comfortable with the environment, a catheter was then inserted into the antecubital vein of the arm. This was kept patent by saline drip throughout the remainder of the testing day. At time -15 min, a fasting blood sample was taken. At +0 min, another blood sample was obtained. Then the participant was provided with the respective test beverage and given 10 min to consume it. Blood samples were completed at +15, 30, 45, 60, 90 and 120 min and the appetite questionnaires were completed right after the blood drawing at each time point. Afterwards, the participants were permitted to leave the facility. This format was repeated for each of the 5 remaining testing days.

4.2.4 Repeated Blood Sampling

Eight blood sample of 4 ml each (32 ml/testing day) were collected throughout the testing day for each participant. The samples were collected in test tubes containing EDTA (ethylenediaminetetraacetic acid). After 10 min storage in an ice bath, the samples were centrifuged at 4°C for 10 min. The plasma was separated and stored in microcentrifuge tubes at

-80°C for future analysis. Plasma glucose was measured with a glucose colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI). Extra plasma was stored at -80°C for future research.

4.2.5 Appetite Questionnaire

Paper questionnaires, assessment of appetite sensations (i.e., hunger, fullness, prospective food consumption, motivation to eat), cravings (thirst, sweet, savory), and overall pleasure/well-being were completed at -15, +0, 15, 30, 45, 60, 90, and 120 minutes throughout the testing period. The questionnaires contained validated visual analog scales (VAS) incorporating a 100 mm horizontal line rating scale for each response (Flint and others 2000). The questions are worded in the following manner “how strong is your feeling of” with anchors of 0 = “not at all” to 100 = “extremely”.

4.2.6 Data and Statistical Analysis

Summary statistics (sample means, sample standard deviations, and area under the curve) were computed for all data. A repeated measures ANOVA was applied to compare main effects of time and treatment on the primary outcomes including plasma glucose concentration as well as subjective appetite. In addition, 2-h area under the curve (AUC) was also calculated for each outcome. Post-hoc analyses were performed using Tukey’s significant test to identify differences among treatments when main effects were detected. Analyses were conducted with Statistical Package for the Social Sciences (SPSS; version 24.0; Chicago, IL). $P < 0.05$ was considered to be statistically significant.

4.3 Results

4.3.1 Postprandial Blood Glucose Response

Figure 8 shows mean deviations from baseline blood glucose for all test beverages. Fasting blood glucose concentration remained consistent prior to treatments at -15 min and 0 min. After the subjects consumed test beverages, their blood glucose levels began to rise and peaked at 30 min for all treatments. The blood glucose concentration of CONT treatment reached 125.3 mg/dL whereas only 102.7 mg/dL for SPI+ALG-6. After reaching their peak value, blood glucose concentrations began to decline and gradually decreased to around the baseline. As shown in Table 4, at 15 min, none of the treatments showed any significant differences in blood glucose concentration. Compared to CONT, postprandial changes in peak glucose concentrations (30 min) were reduced 36.3%, 53.2%, and 58.5% ($P < 0.05$) when subjects consumed SPI-6, SPI+ALG-7, and SPI+ALG-6, respectively. Consumption of SPI-6, SPI+ALG-7, or SPI+ALG-6 also resulted in significantly lower glucose response ($P < 0.05$) at 45 and 60 min compared to CONT. For CONT, blood glucose concentration returned to the basal concentration at 90 min. However, time for blood glucose to return to the baseline was reduced to 60 min for ALG, SPI-7 as well as SPI-6 and 45 min for both SPI+ALG-7 and SPI-ALG-6 ($P < 0.05$).

Examination of the glucose incremental AUC revealed that SPI-6, SPI+ALG-7 and SPI+ALG-6 displayed significantly lower values ($P < 0.05$) of 1-h incremental AUC compared to CONT (Figure 9). Moreover, the AUC for SPI+ALG-6 was significantly lower than that of ALG. For the 2-h incremental AUC (Figure 10), only SPI+ALG-6 was significantly lower than CONT. SPI-6, SPI+ALG-7, and SPI+ALG-6 also showed significantly lower 2-h absolute AUC than CONT ($P < 0.05$, Figure 11).

4.3.2 Appetite

No significant differences were detected in any of the 2-h AUC of appetite profiles among the 6 different treatments. (Refer to Appendix)

Figure 8. Blood glucose response before and after consumption of different types of beverage.

Time 0 is when the beverage was consumed.

Table 4. Change of blood glucose before and after consumption of different types of beverages.

Time	Blood Glucose Change (mg/dL)					
(min)	CONT ^{#§}	ALG	SPI-7	SPI-6	SPI+ALG-7	SPI+ALG-6
-15	0 ^{aA}	0 ^{aA}	0 ^{aA}	0 ^{aA}	0 ^{aA}	0 ^{aA}
0	-0.2 ^{aA}	-0.1 ^{aA}	-0.6 ^{aA}	-0.2 ^{aA}	-0.3 ^{aA}	-0.9 ^{aA}
	(2.2)	(1.9)	(1.6)	(2.1)	(1.6)	(1.7)
15	12.5 ^{abA}	9.6 ^{aA}	10.6 ^{abA}	4.9 ^{abA}	8.0 ^{aA}	4.6 ^{abA}
	(3.3)	(4.0)	(2.3)	(2.1)	(3.4)	(3.4)
30	42.7 ^{dA}	37.0 ^{bAB}	28.4 ^{cABC}	27.2 ^{cBC}	20.0 ^{bC}	17.7 ^{bC}
	(4.0)	(3.4)	(2.8)	(3.6)	(3.0)	(4.8)
45	33.2 ^{cdA}	26.9 ^{bAB}	16.9 ^{bcABC}	15.6 ^{bcBC}	9.0 ^{abC}	5.2 ^{abC}
	(4.7)	(4.3)	(4.1)	(4.6)	(2.7)	(4.2)
60	19.8 ^{bcA}	11.1 ^{aAB}	8.7 ^{abAB}	2.6 ^{aB}	1.1 ^{aB}	1.4 ^{aB}
	(5.1)	(3.6)	(4.2)	(2.6)	(3.7)	(4.2)
90	-1.1 ^{aA}	-1.2 ^{aA}	1.5 ^{aA}	2.9 ^{aA}	-1.1 ^{aA}	0.1 ^{aA}
	(4.1)	(3.3)	(2.5)	(1.7)	(3.1)	(3.4)
120	-2.7 ^{aA}	-1.8 ^{aA}	-0.2 ^{aA}	0.9 ^{aA}	-0.6 ^{aA}	0.7 ^{aA}
	(2.6)	(4.1)	(3.5)	(2.0)	(2.7)	(3.5)

* Data is shown as mean (standard error).

§ Values within a row not sharing an uppercase letter are significantly different.

Values within a column not sharing a lowercase letter are significantly different.

Figure 9. Net incremental area under the curve (AUC) during 1-h beverage tolerance test.

Different letters denote significance ($p < 0.05$) between treatments.

Figure 10. Net incremental area under the curve (AUC) during 2-h beverage tolerance test.

Different letters denote significance ($p < 0.05$) between treatments.

Figure 11. 2-h net absolute area under the curve (AUC) for the different treatments. Different letters denote significance ($p < 0.05$) between treatments.

4.4 Discussion

Previous studies from our laboratory and others have demonstrated that interactions between protein and fibers during *in vitro* gastric digestion could lead to the formation of intragastric gel (Zhang and Vardhanabhuti 2014d; Zhang and others 2014c; Zhang and others 2014b; Hu and others 2017; Borreani and others 2016). This study determined whether consumption of beverages that formed gel when mixed with SGF could result in improved glycemic responses in healthy subjects. The main findings of the study were: 1) SPI-6, SPI+ALG-7 and SPI+ALG-6 all exhibited significantly lowered peak glucose concentrations (30 min) with SPI+ALG-7 and SPI+ALG-6 showing a greater reduction, 2) SPI-6, SPI+ALG-7 and SPI+ALG-6 attenuated in 1-h AUC and 2-h absolute AUC with SPI+ALG-6 also showing a significant reduction in 2-h AUC, and 3) no significant difference was found in any appetite attributes.

Previously, some human and animal clinical studies revealed the potential effect of soy protein on controlling blood glucose levels (Lee 2006; von Post-Skagegård and others 2006; Lang and others 1999; Lavigne and others 2000). Despite lower peak glucose concentration, two SPI beverages in this study were not different from CONT. A possible explanation for this divergent outcome could be the inadequate amount of protein intake in this mixture. Also, the soy protein-based food utilized in previous studies were all in solid form, so the physical state of food could potentially influence the postprandial blood glucose response. According to our previous *in vitro* study, SPI-6 could form a weak intragastric gel once it entered the gastric phase; whereas SPI-7, remained a fluid. Furthermore, part of the sugar in SPI-6 was trapped in the intragastric gel such that it showed lower initial sugar release than SPI-7. In fact, many human clinical studies have successfully illustrated that postprandial blood glucose response correlated with the physical state and structure of food (O'Dea and others 1980; Björck and others 1994; Collier and O'Dea

1982; Granfeldt and others 1994). It is possible that the additional effect of intragastric gel formation of SPI-6 also plays an important role.

Beverages containing mixed SPI and alginate especially at pH 6 were the most effective in lowering postprandial blood glucose concentration. Based on our previous *in vitro* study, mixed SPI and alginate beverages formed well-defined intragastric gel resulting in slower sucrose release. Thus, it is likely that the interaction between SPI and alginate observed in the *in vitro* study is also the mechanism behind the decrease in postprandial blood glucose concentration. Findings from this study support our hypothesis that interactions between SPI and alginate in the gastric environment and potentially the formation of intragastric gel leads to improved postprandial blood glucose.

Previous research revealed the effect of alginate on lowering blood glucose levels in both human subjects (Williams and others 2004; Paxman and others 2008) and animal subjects (Kimura and others 1996). In this study, ALG did not show any significant effect on lowering blood glucose concentration. This is likely due to the low amount of alginate content in the beverages. It has been reported that the effect of fiber on glucose response is dose-dependent (Post and others 2012) and a lower blood glucose response can be found with at least 5 g of fiber consumption (Jenkins and others 2010). The higher viscosity induced by a greater amount of alginate could delay food diffusion in the stomach leading to a lower blood glucose level as a result (Kendall and others 2010; Fabek and others 2014). The high concentration threshold of fibers creates a challenge to incorporate them in food products especially beverages, but, utilizing the gastric interactions between protein and fiber will allow the development of a wider range of products for improved glycemic control.

In this study, no significant difference in appetite profiles was detected among any of the 6 beverages, which indicated an absence of appetite effect compared with CONT. There could be several reasons leading to the insignificant appetite results. First, 12.5 g of protein in the beverages and the energy intake may not provide sufficient protein and energy to sustain satiety throughout the 2-h study. In fact, studies proposed that in order to maintain a satiety state, a diet must be high in protein and provide at least 35-50 g protein and 350-550 kcal energy intake (Leidy and others 2013; Leidy and others 2008). Additionally, at comparable energy intake, liquid food generally provides less satiety compared to solid food. (Tieken and others 2007; Pan and Hu 2011). Though SPI+ALG-7 and SPI+ALG-6 may induce the formation of intragastric gel, the physical properties of the gel are not comparable to those of solid food employed in those studies.

A future study could include the monitoring of postprandial insulin concentration after beverage consumption. This will provide greater insight into understanding the effect of intragastric gelation on blood glucose suppression (Anderson and others 1997; Pyörälä 1979). Additionally, in order to assess satiety, it will be more appropriate to increase protein content per serving and equalize the total energy of the beverages.

4.5 Summary

In summary, consumption of beverages containing SPI and alginate improved postprandial blood glucose response as shown by lower blood glucose concentrations and incremental AUC.

Findings from this study support our hypothesis that interactions between SPI and alginate in the gastric environment and potentially the formation of intragastric gel leads to improved postprandial blood glucose.

CHAPTER 5

CONCLUSION

In this study, we determined the effect of alginate on digestion properties and sucrose release of SPI-based beverages. We found that alginate influenced the physical properties of SPI beverages. Heating SPI and alginate at $\text{pH} > \text{pI}$ resulted in the formation of soluble aggregates with larger size and more negative charges. The size and charge of the aggregates depended on alginate concentration and pH. The addition of alginate also led to beverages with higher pseudoplastic behavior and higher viscosity. Under *in vitro* gastric digestion, digestibility of SPI highly depends on the alginate concentration and, to a lesser extent, pH. Model beverages prepared at pH 6.0 were more resistant to digestion and exhibited slower sucrose release than those at pH 7.0 at the same alginate concentration. Increased alginate concentration resulted in intragastric gel formation that was more resistant to digestion and released sucrose at the slower rate. In the second part of the study, we found that consumption of beverages containing SPI and alginate that formed intragastric gel improved the postprandial blood glucose response as shown by lower blood glucose concentrations and decreased incremental AUC. Findings from the study support our hypothesis that interactions between SPI and alginate in the gastric environment and potentially the formation of intragastric gel leads to improved postprandial blood glucose.

APPENDIX

(a)

(b)

Figure 1. Perceived hunger across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

(a)

(b)

Figure 2. Perceived fullness across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

(a)

(b)

Figure 3. Perceived desire to eat across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

(a)

(b)

Figure 4. Perceived food consumption across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

(a)

(b)

Figure 5. Perceived thirst across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

(a)

(b)

Figure 6. Perceived desire to eat something sweet across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

(a)

(b)

Figure 7. Perceived desire to eat something savory across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

(a)

(b)

Figure 8. Perceived comfort across time for each treatment (a, line graph) and the 2-h incremental AUC for the different treatments (b, bar graph). Time 0 is when the beverage was consumed. Different letters denote significance ($p < 0.05$) between treatments.

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