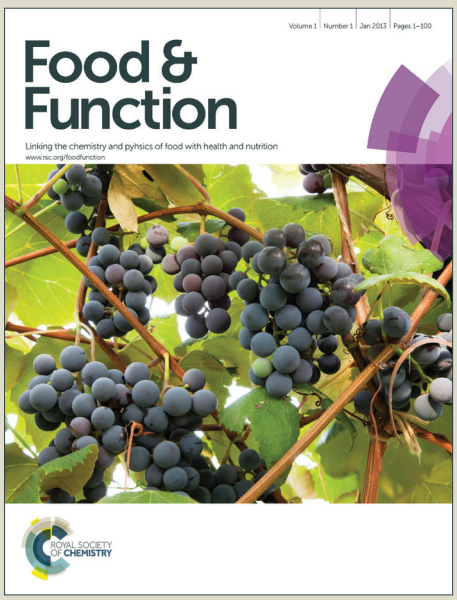
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Effect of charge density of polysaccharides on self-assembled intragastric gelation of whey protein/polysaccharide under simulated gastric conditions

Sha Zhang, Zhong Zhang, Bongkosh Vardhanabhuti*

Food Science Program, Division of Food Systems and Bioengineering, University of Missouri, Columbia, Missouri 65211, United States

*Corresponding author: Tel.: +1 573 882 1374; fax: +1 573 884 7964.

E-mail address: VardhanabhutiB@missouri.edu

Abstract

This study focuses on the behavior of mixed protein and polysaccharides having different charge density under simulated gastric conditions. Three types of polysaccharides, guar gum, xanthan gum and carrageenan (neutral, medium negatively, and highly negatively charged, respectively) were selected to be heated together with whey protein isolate (WPI) at biopolymer ratio ranging from 0.01 to 0.1. Upon mixing with simulated gastric fluid (SGF), all WPI–guar gum samples remained soluble; while WPI–xanthan gum and WPI–carrageenan at biopolymer ratio higher than 0.01 led to self-assembled intragastric gelation immediately after mixing with SGF. The mechanism behind intragastric gelation is believed to be the cross-linking between oppositely charged protein and polysaccharides when pH was reduced to below the pI of the protein. Higher biopolymer ratio led to higher degree of intermolecular interaction, which tends to form stronger gel. More negatively charged carrageenan also formed stronger gel than xanthan gum. SDS-PAGE results show that digestibility of protein was not affected by the presence of guar gum, as well as xanthan gum and carrageenan at biopolymer ratio lower than 0.02. However, intragastric gel formed by WPI–xanthan gum and WPI–carrageenan at biopolymer ratio higher than 0.02 significantly slows down the digestion rate of protein, which could potentially be used to delay gastric emptying and promote satiety.

Key words: intragastric gelation, whey protein, polysaccharides, charge density, digestibility

Introduction

An increased interest in digestion of protein in the gastrointestinal (GI) tract over the recent years is driven by an increase of food related illnesses, such as obesity epidemic and food allergy.[1-3] Protein has been reported to be the most satiating of all micronutrients, which could potentially be used for weight management and obesity control.[4-7] Ingestion of foods evokes satiety in the GI tract by two ways, mechanical stimulation and humoral stimulation.[8] The digestion rate of foods determines the availability of nutrients in the GI tract, which will be sensed and responded by the release of hormonal signals: a delay in gastric emptying may evoke a satiety effect.[9] Hence, the satiety of certain foods may be enhanced by slowing their degradation rate. The digestion rate of protein could be manipulated by various food processing methods through altering the accessibility of the enzymatic cleavage site on protein.[10-12] Native structure of β-lactoglobulin is very resistant to proteolysis, while heating, emulsification, foaming and high pressure treatments led to completely or partially unfolding of protein, exposing more susceptible peptide bonds for enzyme hydrolysis and resulting in enhanced rate of proteolysis.[1, 13-15]

Protein structure could get even more complex as individual proteins can interact with other constituent in food system such as dietary fiber. Dietary fiber itself is a satiating agent due to its unique chemical and physical characteristics, among which, thickening has been associated with prolonged gastric emptying and slower transit time through the small intestine.[16-18] Some viscous fibers are not able to form lumps in the stomach, while other dietary fibers such as alginate, could form lumps in the stomach at concentration higher than critical value, producing large volume that prolongs gastric emptying.[19]

However, our approach, that may well control the rate of food digestion without an adverse effect on the enjoyment of food, is the use of mixture of hydrocolloids that respond by self-structuring to the pH conditions experienced inside the stomach. Previous study conducted in our lab showed that mixture of protein and fiber was able to form intragastric gel at much lower polysaccharide concentrations, though no gelation was observed in single biopolymer system.[20] Liquid that is able to form intragastric gel would require longer transit time in the stomach than regular liquid. Therefore, the sol-gel transition occurred under simulated gastric fluid significantly delayed the digestion rate of protein, and could potentially be used to slow gastric emptying and promote satiety. The mixed protein-fiber samples can be considered as model systems for protein-based beverage.

At pH near or lower than the isoelectric point of the protein complexation between protein and polysaccharide could occur, usually driven by the electrostatic interactions between the two oppositely charged biopolymers.[21, 22] The strength of the attractive interaction depends to a great extent on the macromolecular charge densities.[23-25] It is well demonstrated that higher charged polysaccharides have higher degree of interaction with protein, and sulphated polysaccharides such as carrageenan also interact more strongly with protein than carboxylated polysaccharides such as pectin.[23] Similar to the interactions occurred during complexation, we believe that the mechanism behind intragastric gelation is the electrostatic interaction occurred between positively charged protein and anionic pectin when the mixture undergoes from neutral pH to acid pH under simulated gastric conditions. Hence, it is highly possible that different charged polysaccharides would associate with protein at different extent under simulated gastric

conditions, resulting in forming intragastric gel with different gel strength, which might have different rate of gastric emptying. Alginate is able to form strong or weak gel on exposure to stomach acidic conditions, depending on the types of alginate. It has been reported strong-gelling alginate formed larger volume of lumps in the stomach than weak-gelling alginate by *in vivo* imaging, which also decreased hunger and increased fullness sensed by human subjects.[19]

Accordingly, polysaccharides with different charge density were chosen in this study to investigate the intragastric behavior of protein/polysaccharide mixtures. Guar gum, xanthan gum, and carrageenan with charge density ranging from neutrally charged to highly negatively charged were selected to mix with whey protein before *in vitro* gastric digestion which was carried out in the dissolution apparatus. Whey proteins were chosen not only due to the convincing evidence of whey proteins as satiety-inducing agent, but also because whey proteins are often the preferred source for ready-to-drink protein beverage with excellent nutrition qualities and unique functionalities. Furthermore, since our previous study showed that intragastric gelation only occurred at high pectin to protein biopolymer ratio, a range of biopolymer ratio was chosen to determine the critical ratio needed to form intragastric gel. The rheological properties of the intragastric gel, electrophoresis of the digesta, and microstructure of the gel before and after digestion were used to monitor the digestion pattern.

Materials and methods

Materials

Whey protein isolate (WPI) was kindly donated by Davisco Food International (BiPro, Le Sueur, MN). As stated by the manufacturer, the powdered WPI was constituted of 97.9 wt% protein, 2.1 wt% ash, and 0.3 wt% fat (dry weight basis) and 4.7 wt% moisture (wet weight basis). Guar gum (TIC pretested gum guar 8/22 powder), xanthan gum (100% pure xanthan gum), and carrageenan (FMC viscarin GP 209 F) were provided by TIC Gums (White Marsh, MD), FMC (Philadelphia, PA), and Now Foods (Bloomingdale, IL), respectively. Pepsin with enzyme activity higher than 250 units was obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all of the chemicals used were of analytical grade.

Zeta-potential measurements

Guar gum, xanthan gum, and carrageenan stock solution (1%) was prepared by dissolving in Millipore water at ambient temperature for 2 h under continuous stirring. The stock solutions were diluted to 0.1%, and pH was adjusted to 2.0 and 7.0. Zeta-potential of diluted polysaccharide solutions was measured by dynamic light scattering using the Zetasizer Nano ZS (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, United Kingdom). The zeta-potential values are reported as the average of measurements made on two freshly prepared samples, with three readings made per sample.

Heat treatment of WPI–polysaccharides

Whey protein stock solution (10% w/w) was prepared by dissolving WPI in Millipore water (18.2 MΩ) with continuous stirring for 2 h at ambient temperature. Guar gum, xanthan gum, and carrageenan stock solution (1%) was prepared by dissolving in Millipore water at ambient temperature for 2 h under continuous stirring. Protein and

polysaccharide stock solutions were then kept in the refrigerator (4 ^{o}C) overnight for complete hydration. Stock solutions of WPI and polysaccharides were mixed to obtain 5% w/w protein and polysaccharide to WPI weight ratio ranging from 0.01 to 0.1and their pH was adjusted to 7.0. The mixtures were gently mixed before being heated in a temperature-controlled water bath at 85 ^{o}C for 30 min and cooled using running tap water.

Dissolution Experiments

Dissolution experiments were performed according to Pharmacopoeia official methods using Bio-Dis reciprocating cylinder apparatus 3 (Agilent Technologies, Santa Clara, CA). A digitally controlled water circulation/heater was used to maintain the temperature of the dissolution media at 37 ± 0.5 ^{o}C. The dissolution media consisted of 0.034 M NaCl, and 3.2 mg/g pepsin at pH 1.2. Pepsin solution was prepared freshly for each assay by dissolving pepsin in SGF by vortexing several times over a period of 5 min. The dissolution experiments were performed at a reciprocating rate of 20 dips per minute (dpm) using mesh screens of 405 μm mesh size. The dissolution outer tubes were filled with 78 mL of dissolution media, and 10 g of WPI–polysaccharide mixture was added in the inner tube (Supplemental Figure 1). The ratio of pepsin to WPI was 1:2 on a weight basis. Samples (2 mL) for electrophoresis were taken manually from outer tube at time intervals of 2, 5, 10, 20, 30, and 60 min and replenished with 2 mL of fresh dissolution media. In order to control sampling time, the inner tube was positioned above the dissolution media for 0.5 min during sampling. Sodium hydroxide (1 N and 0.1 N) was added to samples to adjust pH to above 7.0 to inactivate enzymes, and DI water was

added to adjust the total volume of the sample to 2.5 mL. Samples were diluted to 1 mg/mL pepsin for electrophoresis analysis.

Swelling Ratio

The intragastric gels of WPI–xanthan gum and WPI–carrageenan at biopolymer ratio of 0.1 were used to characterize the swelling ratio. The weight of the intragastric gels during dissolution was measured to calculate the swelling ratio during digestion with pepsin, in comparison to the swelling ratio measured without pepsin, which was conducted by forming intragastric gel in a sitting beaker. The swelling ratio was determined using the following equation [26]:

$$\text{Swelling ratio} = 100 \times (m_f - m_i)/m_i$$

Where m_f is the final weight of the gel, and m_i is the initial weight of the gel.

Rheological properties

Rheological properties of the WPI–polysaccharide solution after mixing with SGF were measured on a Kinexus rheometer (Malvern Instruments Ltd., Worcestershire, United Kingdom) with a upper plate geometry (20 mm diameter). We used syringe to inject 2.5 mL of WPI–polysaccharide solution to SGF to form a large gel piece with diameter around 20 mm (Supplemental Figure 2). Although intragastric gelation occurred immediately after samples were mixed with SGF, it takes some time to form a uniform gel since the pH of the gel inside decreased slowly, especially when we use syringe to form much larger gel piece than in the dissolution experiment. Hence, the gels were left in the SGF overnight before rheological measurement in order to obtain pH equilibrium.

The next day, gel was cut into a cylinder shape with diameter around 20 mm and height around 2 mm. A gap of 2 mm was used and samples were evenly distributed over the entire surface area of the plate. The elastic modulus (G′) and viscous modulus (G″) was monitored in the pre-determined linear viscoelastic region (0.5% strain) at a constant frequency of 1 Hz and 25 ^{o}C. A strain sweep test was performed subsequently to check that measurements have been done within the linearity limits of the viscoelastic behavior.

Electrophoresis

SDS-PAGE was carried out using a modification of Laemmli method. Samples were solubilized in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 5% β-mercaptoethanol and heated at 95 ^{o}C for 5 min. The samples were cooled to room temperature and loaded (10 μL) onto the gel containing 15% acrylamide for the resolving gel and 4% acrylamide for the stacking gel. The gel was run in a mini Protein II electrophoresis system (Bio-Rad Laboratories) using an electrode stock buffer at a voltage of 120 V. The gels were stained with Coomassie brilliant blue R250 in an acetic acid:methanol:H_2O staining solution (1:4:5 by volume), and destained in an acetic acid:methanol:H_2O solution (1:4:5 by volume). Unstained molecular weight marker comprising a mixture of protein ranging in size from 5 to 250 kDa was used (PageRuler unstained broad range protein ladder: Thermo Scientific, Rockford, IL). Imaging was accomplished with AlphaImager system (Alpha Innotech Corporation, Santa Clara, CA).

Scanning electron microscopy

After mixing with SGF, WPI–carrageenan gelled immediately, and a small piece of the gel was taken out and put into NaOH solution to inactivate the enzymes, which represent

the initial microstructure of the gel. The microstructure of the gel after digestion was also monitored. After the gels were digested in the dissolution apparatus for 1 h, gel pieces became smaller, and one piece of the gel was taken and directly put into NaOH solution to inactivate the enzymes. Both initial gel and digested gel specimens were then fixed in 2% glutaraldehyde 2% paraformaldehyde/0.1 M sodium cacodylate buffer solution at 4 ^{o}C overnight, followed by rinsing in 0.1 M sodium cacodylate buffer for three times (15 min each). Washed gel specimens were dehydrated in a series of aqueous ethanol solutions ranging from 30% to 100%. Dehydrated specimens were critical point dried, mounted on aluminium stubs and coated with 10 nm of platinum using a Sputter Coater (EMS575X, Electron Microscopy Sciences, Hatfield, PA). SEM studies were carried out using a FEI Quanta 600 F (FEI Company, Hillsboro, OR) extended vacuum scanning electron microscope. In all cases, acceleration voltage of 10 kV was used. Digital micrographs, acquired at magnification ranging between × 3000 and × 50000 were captured.

Results

Intragastric gelation

In order to verify that the charge density of polysaccharides used in this study ranges from low to high, zeta-potential of polysaccharide solutions were measured at both pH 7.0 and 2.0 (Table 1), which were the representative pH values of WPI–polysaccharides before and after mixing with SGF, respectively. Guar gum is usually recognized as a neutral polysaccharide, and it is generally unaffected by pH changes or an increase in other ionic species.[27] On the other hand, xanthan gum and λ-carrageenan are negatively

charged polysaccharides under a wide range of pH values, and the pH of the medium has a great impact on their charge density due to the protonation of the carboxyl groups. As shown in Table 1, the zeta-potential of guar gum changed from -8.19 mV to 2.88 mV when pH was reduced to 2.0, indicating that guar gum used in this study carried little negative charges at neutral pH, while the amount of the negative charges decreased even further at acidic pH. For negatively charged xanthan gum and carrageenan, reduction of pH from 7.0 to 2.0 also decreased the amount of the negative charges. However, these two macromolecules remain negatively charged at acidic pH, with carrageenan carrying more negative charges than xanthan gum.

WPI and polysaccharides at biopolymer ratio ranging from 0.01 to 0.1were mixed with SGF in the test tube to show the intragastric behavior of the biopolymers. As shown in Figure 1, the ability of the mixtures to form intragastric gel depends on both the nature of polysaccharides and the biopolymer ratio. At all biopolymer ratios studied, WPI–guar gum did not form gel and remained soluble after mixing with SGF, and no difference in the turbidity of the mixture was observed (Figure 1 A–D). For both xanthan gum and carrageenan, no intragastric gel was observed at lowest biopolymer ratio of 0.01 (Figure 1 E and I), while increasing biopolymer ratio to 0.02 led to the formation of lump in SGF (Figure 1 F and J), and further increasing biopolymer ratio resulted in extensive gelation immediately after mixing with SGF (Figure 1 G–H and K–L). It should be noted that no gelation occurred when single biopolymer was mixed with SGF. Furthermore, WPI–carrageenan seems to form more turbid and denser gel than WPI–xanthan gum.

Rheological properties of WPI–polysaccharides after mixing with SGF were measured using frequency sweep (Figure 2). For all guar gum-contained samples, the loss modulus,

230 G″, was dominant over the storage modulus, G′, indicating no gel formation (data not
231 shown). For samples containing xanthan gum and carrageenan at biopolymer ratio of
232 0.01, G″ was dominant over G′, indicative of a liquid-like response, while G′ was
233 dominant over G″ for xanthan gum and carrageenan samples at biopolymer ratios higher
234 than 0.01, indicating a gel-like material response. For all gel-like samples, both G′ and G″
235 exhibited a weak frequency dependence within the frequency range used in this study.
236 Furthermore, for both WPI–xanthan gum and WPI–carrageenan, the elastic moduli
237 increased with increasing biopolymer ratio, suggesting that the presence of higher amount
238 of polysaccharides promoted the degree of cross-linking between protein and
239 polysaccharide molecules, thus forming gel with enhanced gel strength. It should also be
240 noted that at the same biopolymer ratio, WPI–carrageenan gel was stronger than WPI–
241 xanthan gum gel, which was consistent with our visual observation (Figure 1). Stronger
242 gel shown in samples with carrageenan was likely due to its higher charge density, as
243 discussed below.

244 Schematic illustrations of intragastric gelation of WPI–polysaccharides with different
245 charge density are shown in Figure 3. Protein molecules unfold and aggregate to form
246 large aggregates upon heating together with polysaccharides at neutral pH. Due to the
247 strong repulsion between biopolymers, the electrostatic interaction between protein and
248 polysaccharides is very limited despite the charge density of the polysaccharides. When
249 WPI–polysaccharide solution is mixed with SGF where the pH is reduced to far below
250 the pI of the protein, protein immediately becomes highly positively charged. This
251 immediate charge reversal of protein allows interactions between the biopolymers. For
252 neutral polysaccharides, there are no charged groups available to interact with the

positively charged groups of protein, hence, polysaccharides and protein remain co-soluble in SGF. In contrast, as shown by zeta-potential results, negatively charged polysaccharides still maintain negatively charged properties after mixing with SGF; thus, the electrostatic interactions between carboxylic groups of polysaccharides and the amino group of protein could occur, leading to the cross-linking of the biopolymers. Low polysaccharide to protein ratio is not sufficient for the biopolymers to form interconnected gel network. Increasing biopolymer ratio increases the degree of cross-linking to such an extent that the inter-biopolymer attractions lead to gel network formation. Higher biopolymer ratio is expected to have higher degree of inter-biopolymer interaction, which leads to the formation of gel with higher strength. Similar mechanism could be used to explain polysaccharides with different charge density. At the same biopolymer ratio, higher charged polysaccharides are expected to have higher degree of association with protein. As a result, a strong structure is created rather than a weak one.

Dissolution experiments

We previously reported that the intragastric gelation significantly slowed down the digestion rate of protein for whey protein and pectin system under simulated gastric conditions. In this work, the digestion pattern of protein was evaluated using dissolution experiment. Dissolution apparatus is commonly used in pharmaceutical industry to provide *in vitro* drug release information.[28] It has also been used to study the release of minerals and bioactive components from protein hydrogels.[29] Since some samples formed strong gels upon mixing with SGF, the digestion of protein–polysaccharide solution turned into digestion of protein–polysaccharide gel. Hence, dissolution apparatus is an appropriate means to study the intragastric gelation and would provide important

information about the degradation of the gel and release of the protein and peptides from the gel.

Swelling ratio

Since WPI–xanthan gum and WPI–carrageenan at biopolymer ratio of 0.1 formed strong gels, these two samples were selected to monitor the swelling ratio. Other samples did not form gel or formed weak gels, making it difficult to measure the weight of the gel during digestion. Figure 4 shows the swelling behavior of these two gels with and without pepsin. In the absence of pepsin, gels with xanthan gum and carrageenan followed different trend: WPI–xanthan gum gels swelled in the first 30 min and then the weight kept constant; WPI–carrageenan only swelled somewhat in the first 5 min and then the weight of the gel slightly decreased. Although these two samples formed intragastric gels immediately when mixed with SGF, the inside of the gels remained liquid at first since it takes some time for the pH of the whole mass to reach the equilibrium. The possible reason for the shrinking of WPI–carrageenan gels after 5 min is that the penetration of the protons into the inside of the gel resulted in the decrease in the repulsive charges, which allowed protein and carrageenan molecules to come closer together and form network. The same phenomenon could happen for WPI–xanthan gum gel; however, the higher swelling ratio could be due to the different microstructural feature of the gels, which will be discussed later under the Microstructure section. In the presence of pepsin, the weight of the two gels rapidly decreased in the first 10 min of digestion, but the decrease became slower in the following 50 min, especially for WPI–xanthan gum. There were still 69.8% and 38.3% of gel remained undigested after 1 h for WPI–xanthan gum and WPI–carrageenan, respectively.

SDS-PAGE

During dissolution experiments, samples were also periodically taken and the *in vitro* digestion patterns of WPI–polysaccharides were examined using SDS-PAGE. Figure 5 shows the analysis of digesta from WPI–guar gum with biopolymer ratio ranging from 0.01 to 0.1. These four samples show similar proteolysis pattern during simulated gastric digestion, with the appearance of dense peptide bands that decreased with time. This revealed that biopolymer ratio did not affect the digestion pattern of protein. It has been shown that digestibility of protein depends on the degree of the denaturation. Heating WPI resulted in the unfolding of protein and exposure of peptide bonds, which were susceptible to pepsin cleavage. Protein that remained in its native state after heating was very resistant to pepsin. With the high pepsin to protein ratio used in this work, the majority of the denatured protein was broken down to smaller peptides within 2 min, showing several intensive peptide bands on SDS-PAGE (Figure 5, lane 4). Only light β-lg band was observed, corresponding to the β-lg remaining in its native state after heating. The unchanged β-lg band during further digestion was consistent with previous reports, indicative of the resistance of native β-lg to pepsin digestion. The most intense peptide bands were observed at 2 min, and they became lighter along the digestion since they were degraded into peptides with lower molecular masses or even amino acids, which could not be shown on the gel. As the digestion time was increased up to 1 h, only faint bands of peptides were detected.

For WPI–xanthan gum and WPI–carrageenan, which formed intragastric gel with high biopolymer ratio, the digestion pattern of protein was significantly affected by its biopolymer ratio. As stated previously, samples with lowest biopolymer ratio (0.01) did

not gel when mixed with SGF. The digestion pattern of these samples was very similar to the one with guar gum, indicating that addition of xanthan gum or carrageenan at lowest biopolymer ratio did not affect the digestibility of protein (Figure 6A and 7A). Although increasing biopolymer ratio to 0.02 led to gel-like structure formation as shown by its rheological properties, such weak gel was degraded by SGF very fast. From our visual observation, gels were all dissolved in the SGF within 2 min of digestion; from the SDS-PAGE, it can be seen that the proteolysis of protein was the same as the one with biopolymer ratio of 0.01 (Figure 6B and 7B).

Higher biopolymer ratio remarkably reduced the degradation rate of protein. For WPI–xanthan gum at biopolymer ratio of 0.05 and 0.1, the intensity of the bands shown at 2 min digestion was much weaker compared to the one with lower biopolymer ratio. The decrease in the intensity of the peptide bands was observed along the digestion up to 1 h, which was consistent with the results from weight change, indicating the decreased digestion rate over time. In contrast, there seems to be a slight increase in the band intensity with time for WPI–carrageenan having biopolymer ratios of 0.05 and 0.1. Interestingly, although WPI–xanthan gum showed higher weight remaining than WPI–carrageenan through digestion, the peptide bands shown on WPI–carrageenan were much less intense. This suggests that WPI-xanthan gel absorbed larger amount of water (higher degree of swelling) but the protein was digested faster. On the other hand, WPI–carrageenan gel absorbed less amount of water than WPI–carrageenan, probably due to its compact and dense gel network and was digested slower.

Microstructure

WPI–xanthan gum and WPI–carrageenan with highest biopolymer ratio was chosen as the examples to reveal the initial gel microstructures and the microstructure of the gel after digestion (Figure 8). The spherical particles shown on SEM images are protein aggregates, while the fibril filaments were polysaccharides. Figure 8 (a) and (c) shows the initial gel microstructure of WPI-xanthan gum and WPI-carrageenan, respectively. WPI–xanthan gum gel consisted of filamentous network where protein aggregates tended to form clusters and seemed to interact with the filamentous network. WPI–carrageenan gel exhibited microstructural features that were significantly different from WPI–xanthan gum. No clear sign of carrageenan was visible on the gel; however, some of the protein aggregates were assembled in linear shape, indicating that carrageenan was buried in the protein aggregates. Furthermore, protein aggregates in WPI–carrageenan gels formed much larger clusters than WPI–xanthan gum, likely because of the stronger attraction between protein aggregates and carrageenan.

For both WPI–xanthan gum and WPI–carrageenan gels digested for 1 h, the protein aggregates that attached onto the polysaccharides were partially broken down and more filaments were exposed on the surface of the gel (Figure 8 (b) and (d)). From our visual observation, the gel pieces were getting smaller and smaller during digestion. This is because protein was gradually removed from the gel network by the activity of pepsin. Without the attached protein, polysaccharides were eventually dissolved into the digestion medium, resulting in the decrease in the gel size.

The microstructure of the gel also explained the different swelling ratio observed between the two gels. Although protein aggregates and carrageenan were strongly associated in the local area, the overall feature of WPI–carrageenan gel show large pore size. WPI–

xanthan gum gel has lower density of protein aggregates and much smaller pores evenly distributed in the gel network, which tends to hold more water, resulting in higher swelling ratio during dissolution.

Discussion

The results from dissolution experiment clearly showed that the digestion of protein in the presence of neutral polysaccharides, which did not form intragastric gel, was not affected by the addition of the polysaccharides. On the contrary, addition of negatively charged polysaccharides could significantly slow the digestion rate of protein by intragastric gelation, depending on the biopolymer ratio of polysaccharides to protein. Guar gum, as a neural polysaccharide, has very limited interaction with protein during heating at neutral pH. When mixed with SGF, the two macromolecules remained co-soluble and did not interact with each other. The presence of guar gum during gastric digestion did not influence the accessibility of pepsin to susceptible peptide bonds of protein, hence, the digestibility of the protein was not affected by the concentration of guar gum.

Negatively charged xanthan gum and carrageenan also had very limited interaction with protein during heating at neutral pH due to the repulsion between biopolymers, however, positively charged protein associated with negatively charged polysaccharides upon mixing with SGF. Although it has been reported that negatively charged polysaccharides could decrease protein digestibility by interaction with some protein molecules,[30-32] the proteolysis of protein was not affected by the polysaccharides in this study, probably due to the high pepsin to protein ratio used, which rapidly degraded protein within 2 min.

Even though protein and polysaccharides formed lump at biopolymer ratio of 0.02, it was disassociated by physical movement and high concentration of pepsin within 2 min of dissolution. At higher biopolymer ratios, when there were enough polysaccharides to associate with protein molecules and form cross-linked network, the accessibility of peptide bonds on protein was significantly reduced. The majority of the protein was buried inside the gel network, and only the protein on the surface of the gel was accessible to pepsin. It is also possible that the susceptibility of the protein on the gel surface could be reduced due to the interaction with polysaccharides.

The digestibility of the intragastric gel was affected by the strength of the gel. Gel with higher strength usually indicates higher degree of association between protein and polysaccharides. The nature of the association is mainly driven by the electrostatic attraction between oppositely charged biopolymers, which could limit the accessibility of the peptide bond to proteolysis. Therefore, higher charged polysaccharides were expected to have higher degree of interaction with protein which resulted in stronger gel formation and less number of accessible sites for pepsin, leading to slower digestion rate of protein. At the same biopolymer ratio, gels with xanthan gum were weaker than the one with carrageenan; correspondingly, more peptides were detected during the digestion of gels with xanthan gum. In addition, in samples containing lower charged polysaccharides, there might be more dissociative protein that was not involved in the intragastric gelation than in samples with higher charged polysaccharides. These protein molecules were very easy to be digested by pepsin. This could be the reason that more peptides were detected at 2 min of digestion for WPI–xanthan gum.

411 The results obtained in this study indicate that intragastric gelation can be controlled by
412 variations in the types of polysaccharides and the biopolymer ratio of polysaccharides to
413 protein. Manipulation of the protein and polysaccharide mixture could be potentially used
414 to promote satiety. Polysaccharides have been widely used in the food industry as
415 thickener, stabilizer and emulsifier to modify the viscosity, texture, and mouth-feel of
416 food. The presence of negatively charged polysaccharides, not restricted to xanthan gum
417 and carrageenan, in protein-containing meals could lead to extensive coalescence,
418 flocculation or gelation with proteins in the stomach. Several studies indicated that the
419 physicochemical properties of the meal have a great effect on satiety, and meals
420 containing solids typically induced greater satiety than liquid meals with equivalent size
421 and energy content.[19, 33] Therefore, one would expect that the gelation in the stomach
422 could result in a slower initial emptying of the stomach, which will then be sensed as
423 prolonged feeling of fullness. However, the formation of intragastric gel and gel strength
424 will depend upon the physiologic conditions, e.g. rate of acidification, presence of other
425 biopolymers, and ionic concentration. Whether the intragastric gelation could enhance the
426 feeling of fullness *in vivo* is the subject of ongoing study in our lab.

427 **Conclusion**

428 Effect of polysaccharides with different charge density on intragastric gelation of WPI–
429 polysaccharides under simulated gastric conditions has been investigated. The
430 mechanism behind intragastric gelation is believed to be the cross-linking between
431 positively charged protein and negatively charged polysaccharides due to the electrostatic
432 attraction occurred when pH was reduced to below the pI of the protein. Guar gum, as a
433 neutral polysaccharide, has limited interaction with protein; hence, the biopolymers

remained co-soluble upon mixing with SGF, while samples containing negatively charged xanthan gum and carrageenan formed could intragastric gel depending on the biopolymer ratio. At low biopolymer ratio (0.01), no gelation was observed and digestibility of protein was not affected by the presence of the polysaccharides. Higher biopolymer ratio led to extensive intragastric gelation, which significantly slowed down the digestion rate of protein. Intragastric gel with lower charged xanthan had higher degree of swelling but was digested faster compared to that with higher charged carrageenan. Higher degree of interactions between WPI and highly charged carrageenan led to denser intragastric gel with slowest digestion rate.

Acknowledgment

The authors would like to thank Davisco Foods International, TIC Gums, and FMC for providing whey protein isolate (BiPro), guar gum and carrageenan, respectively.

References

1. A. Mackie and A. Macierzanka, *Curr. Opin. in Colloid. In.*, 2010, 15, 102-108.
2. M. Wickham, R. Faulks and C. Mills, *Mol. Nutr. Food Res.*, 2009, 53, 952-958.
3. S. J. Hur, B. O. Lim, E. A. Decker and D. J. McClements, *Food Chem*, 2011, 125, 1-12.
4. M. Veldhorst, A. Smeets, S. Soenen, A. Hochstenbach-Waelen, R. Hursel, K. Diepvens, M. Lejeune, N. Luscombe-Marsh and M. Westerterp-Plantenga, *Physiol. Behav.*, 2008, 94, 300-307.
5. D. Paddon-Jones, E. Westman, R. D. Mattes, R. R. Wolfe, A. Astrup and M. Westerterp-Plantenga, *Physiol. Behav.*, 2008, 87, 1558S-1561S.

6. A. Astrup, *Am. J. Clin Nutr.*, 2005, 82, 1-2.

7. T. L. Halton and F. B. Hu, *J. Am. Coll. Nutr*, 2004, 23, 373-385.

8. M. C. P. Geraedts, F. Troost and W. Saris, *Obe Rev*, 2011, 12, 470-477.

9. R. Capasso and A. A. Izzo, *J. Neuroendocrinol.*, 2008, 20, 39-46.

10. K. Takagi, R. Teshima, H. Okunuki and J.-i. Sawada, *Biol.Pharm. Bull.*, 2003, 26, 969-973.

11. M. Zeece, T. Huppertz and A. Kelly, *Innov. Food Sci. Emerg. Technol.*, 2008, 9, 62-69.

12. Y. Morisawa, A. Kitamura, T. Ujihara, N. Zushi, K. Kuzume, Y. Shimanouchi, S. Tamura, H. Wakiguchi, H. Saito and K. Matsumoto, *Clin. Exp. Allergy*, 2009, 39, 918-925.

13. A. Malaki Nik, A. J. Wright and M. Corredig, *J. Colloid Interface Sci.*, 2010, 344, 372-381.

14. J. Maldonado-Valderrama, A. P. Gunning, P. J. Wilde and V. J. Morris, *Soft Matter*, 2010, 6, 4908-4915.

15. A. Macierzanka, A. I. Sancho, E. C. Mills, N. M. Rigby and A. R. Mackie, *Soft Matter*, 2009, 5, 538-550.

16. M. Kristensen and M. G. Jensen, *Appetite*, 2011, 56, 65-70.

17. J. Slavin and H. Green, *Nutr. Bull.*, 2007, 32, 32-42.

18. N. C. Howarth, E. Saltzman and S. B. Roberts, *Nutr. Rev*, 2001, 59, 129-139.

19. C. L. Hoad, P. Rayment, R. C. Spiller, L. Marciani, B. de Celis Alonso, C. Traynor, D. J. Mela, H. P. Peters and P. A. Gowland, *J. Nutr.*, 2004, 134, 2293-2300.

20. S. Zhang and B. Vardhanabhuti, *Food Funct.*, 2013.

21. S. Turgeon, C. Schmitt and C. Sanchez, *Curr. Opin. in Colloid. In.*, 2007, 12, 166-178.

22. C. Schmitt and S. L. Turgeon, *Adv. Colloid Interface Sci.*, 2011, 167, 63-70.

23. J.-L. Doublier, C. Garnier, D. Renard and C. Sanchez, *Curr. Opin. in Colloid. In.*, 2000, 5, 202-214.

24. A. Ye, *Int. J. Food Sci. Technol.*, 2008, 43, 406-415.

25. B. L. Sperber, H. A. Schols, M. A. Cohen Stuart, W. Norde and A. G. Voragen, *Food hydrocolloid*, 2009, 23, 765-772.

26. P. Rayment, P. Wright, C. Hoad, E. Ciampi, D. Haydock, P. Gowland and M. F. Butler, *Food Hydrocolloid*, 2009, 23, 816-822.

27. Q. Wang, P. Ellis and S. Ross-Murphy, *Food Hydrocolloid*, 2000, 14, 129-134.

28. P. Costa and J. M. Sousa Lobo, *Eur. J. Pharm. Sci.*, 2001, 13, 123-133.

29. G. E. Remondetto, E. Beyssac and M. Subirade, *J. Agric. Food Chem.*, 2004, 52, 8137-8143.

30. A. Nacer S, C. Sanchez, C. Villaume, L. Mejean and J. Mouecoucou, *J. Agric. Food Chem.*, 2004, 52, 355-360.

31. J. Mouécoucou, C. Sanchez, C. Villaume, O. Marrion, S. Frémont, F. Laurent and L. Méjean, *J. Dairy Sci*, 2003, 86, 3857-3865.

32. N. Polovic, M. Blanusa, M. Gavrovic-Jankulovic, M. Atanaskovic-Markovic, L. Burazer, R. Jankov and T. C. Velickovic, *Clin. Exp. Allergy*, 2007, 37, 764-771.

33. L. Marciani, P. A. Gowland, A. Fillery-Travis, P. Manoj, J. Wright, A. Smith, P. Young, R. Moore and R. C. Spiller, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 2001, 280, G844-G849.

505 Table 1. Zeta potential of guar gum, xanthan gum and carrageenan at concentration of
506 0.1% and pH 7.0 and 2.0.

Polysaccharides	Zeta-potential at pH 7.0 (mV)	Zeta-potential at pH 2.0 (mV)
Guar gum	-8.19	2.88
Xanthan gum	-58.2	-22.1
Carrageenan	-83.4	-53.0

507

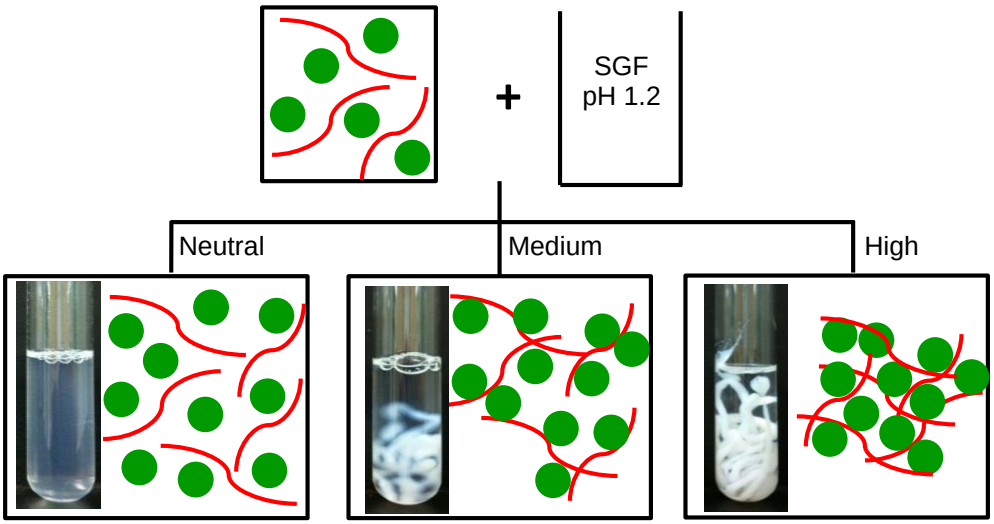
508

509

"for Table of Contents use only"

Effect of charge density of polysaccharides on self-assembled intragastric gelation of whey protein/polysaccharide under simulated gastric conditions

Zhang, Zhang & Vardhanabhuti.



Charge density of polysaccharides and biopolymer ratio are the major factors affecting their intragastric gelation and their digestion properties.

Figure 1. Intragastric gelation of WPI–polysaccharides mixed with SGF: (A – D) guar gum to WPI weight ratio of 0.01, 0.02, 0.05, and 0.1; : (E – H) xanthan gum to WPI weight ratio of 0.01, 0.02, 0.05, and 0.1; : (I – L) carrageenan to WPI weight ratio of 0.01, 0.02, 0.05, and 0.1.

Figure 2. . Elastic modulus (G′) (solid) and Viscous modulus (G″) (empty) of intragastric gel formed by WPI–xanthan gum mixed with SGF (a) and WPI–carrageenan mixed with SGF (b) with different polysaccharide to WPI weight ratio of 0.01, 0.02, 0.05, and 0.1.

Figure 3. Schematic illustrations of intragastric gelation of WPI and polysaccharide with different charge density.

Figure 4. Swelling ratio of WPI–xanthan gum (●) and WPI–carrageenan (▲) intragastric gels during dissolution without (filled) and with (empty) pepsin.

Figure 5. SDS-PAGE profile of in vitro digestion of WPI–guar gum with different guar gum to WPI weight ratio: (A) 0.01; (B) 0.02; (C) 0.05; (D) 0.1; lane 1, standard marker; 2, WPI; 3, pepsin; 4 – 9, digested for 2, 5, 10, 20, 30, and 60 min.

Figure 6. SDS-PAGE profile of in vitro digestion of WPI–xanthan gum with different xanthan gum to WPI weight ratio: (A) 0.01; (B) 0.02; (C) 0.05; (D) 0.1; lane 1, standard marker; 2, WPI; 3, pepsin; 4 – 9, digested for 2, 5, 10, 20, 30, and 60 min.

Figure 7. SDS-PAGE profile of in vitro digestion of WPI–carrageenan with different carrageenan to WPI weight ratio: (A) 0.01; (B) 0.02; (C) 0.05; (D) 0.1; lane 1, standard marker; 2, WPI; 3, pepsin; 4 – 9, digested for 2, 5, 10, 20, 30, and 60 min.

Figure 8. SEM images of WPI–xanthan gum (a and b) and WPI–carrageenan (c and d) (polysaccharides : WPI weight ratio of 0.1) immediately mixed with SGF (a and c) and after 1 h digestion (b and d).

Figure 1.

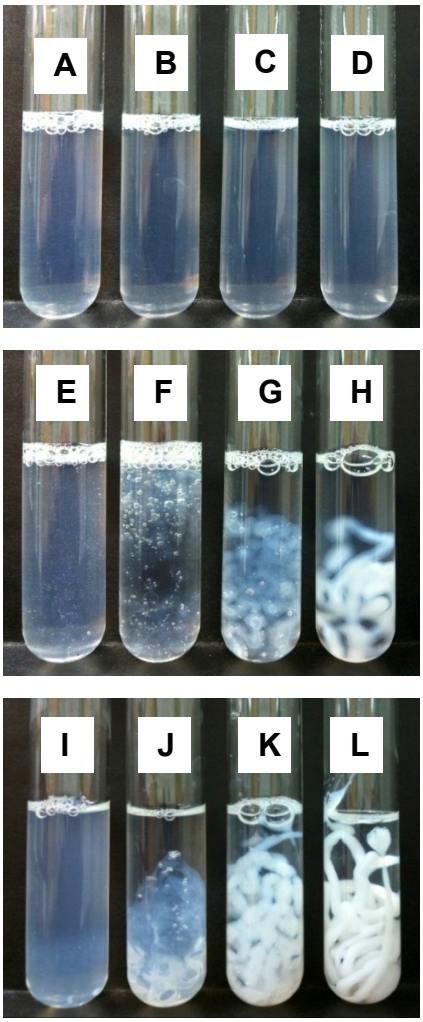


Figure 2.

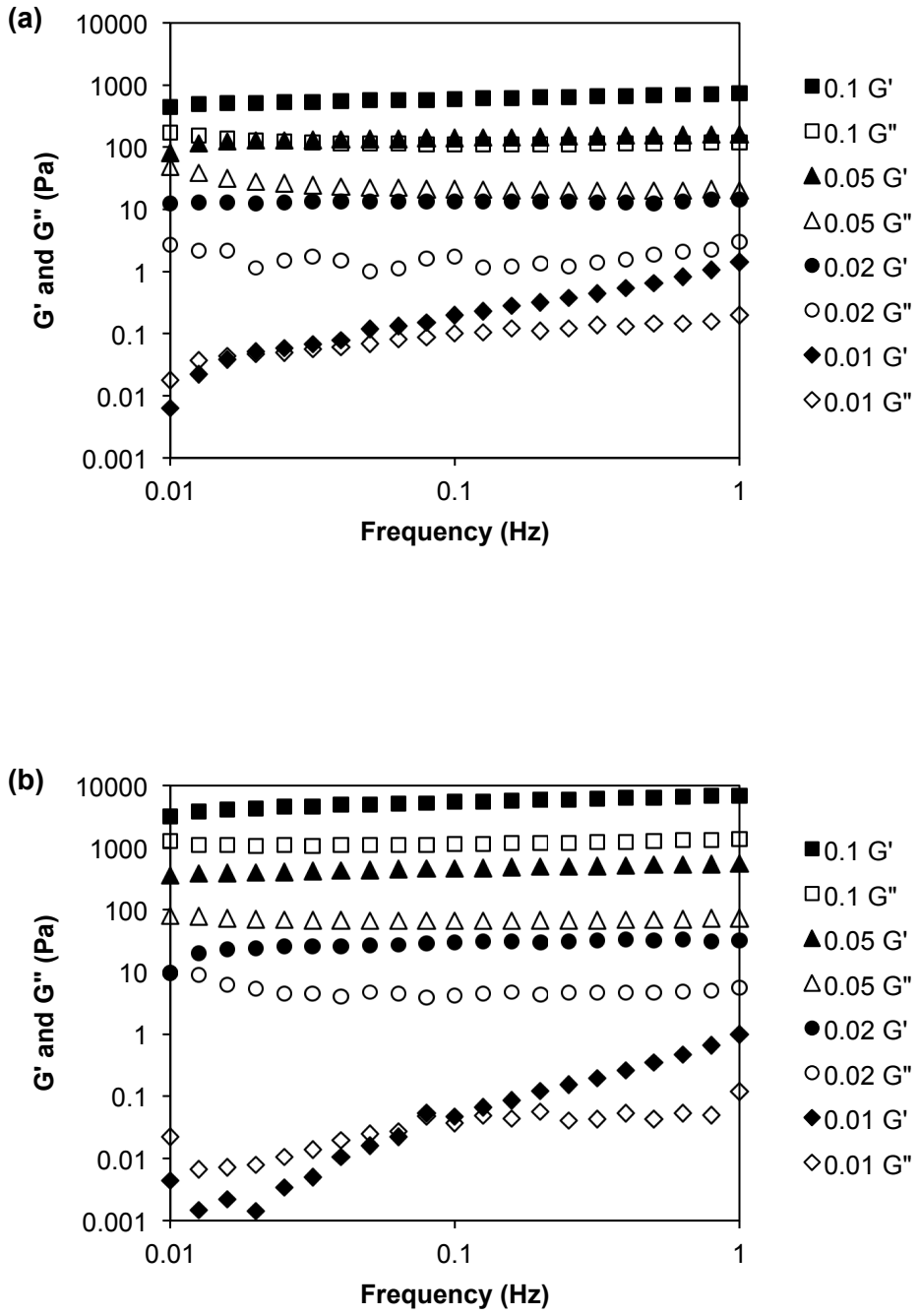


Figure 3.

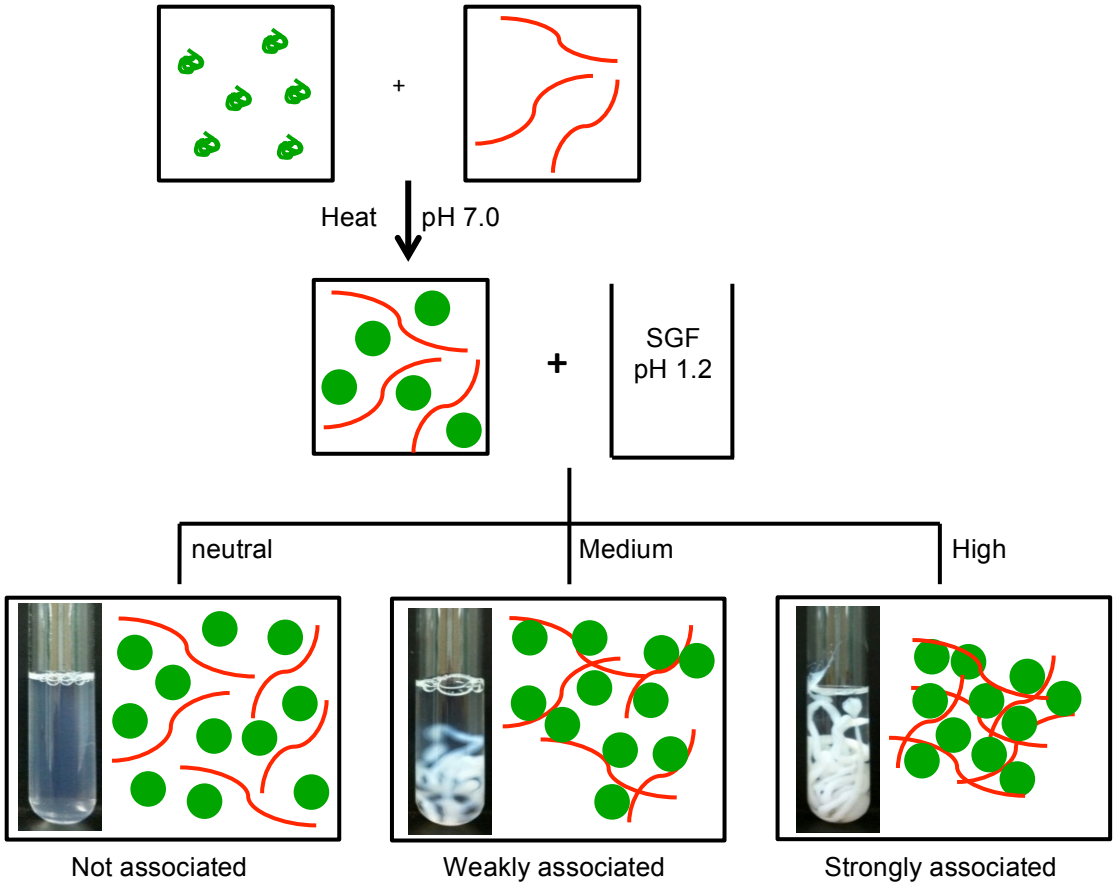


Figure 4.

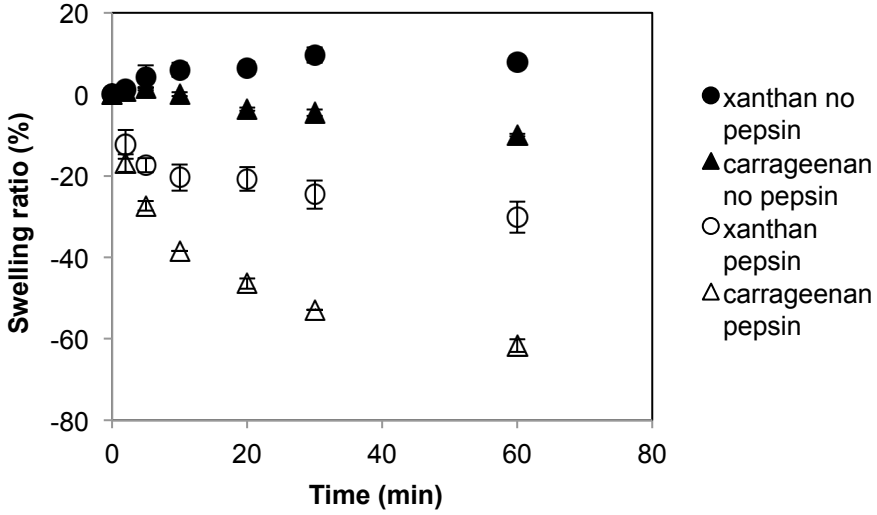


Figure 5.

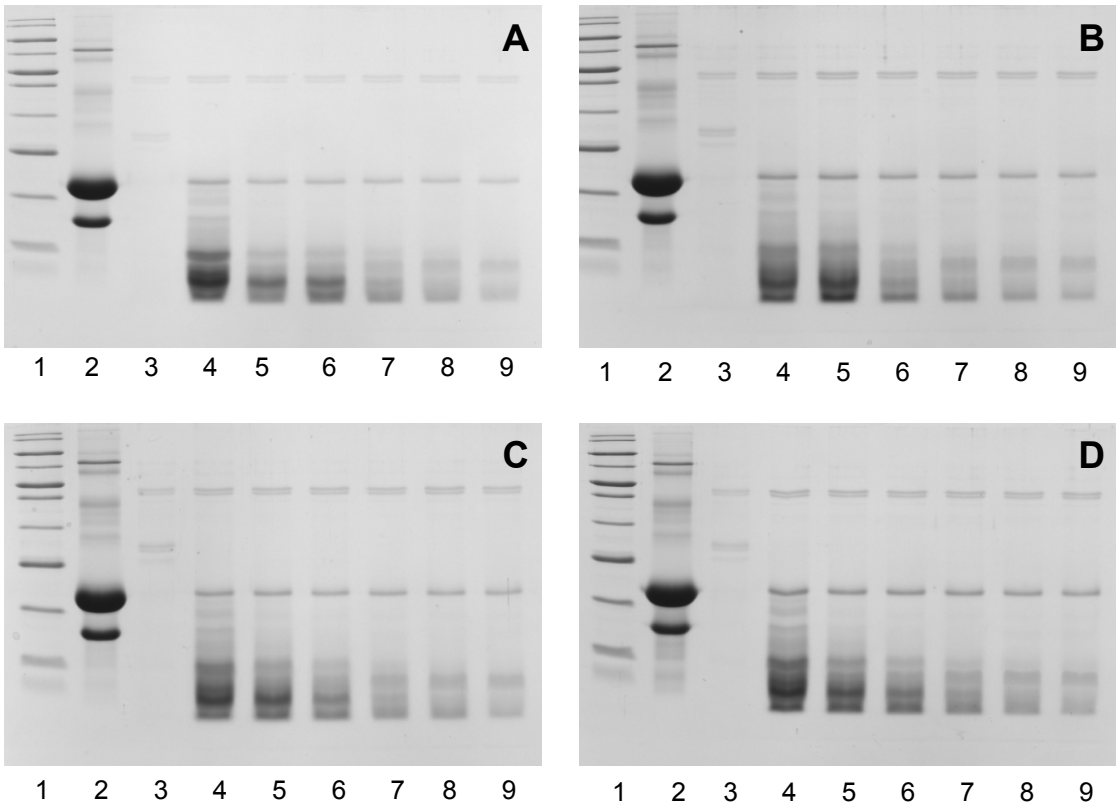


Figure 6.

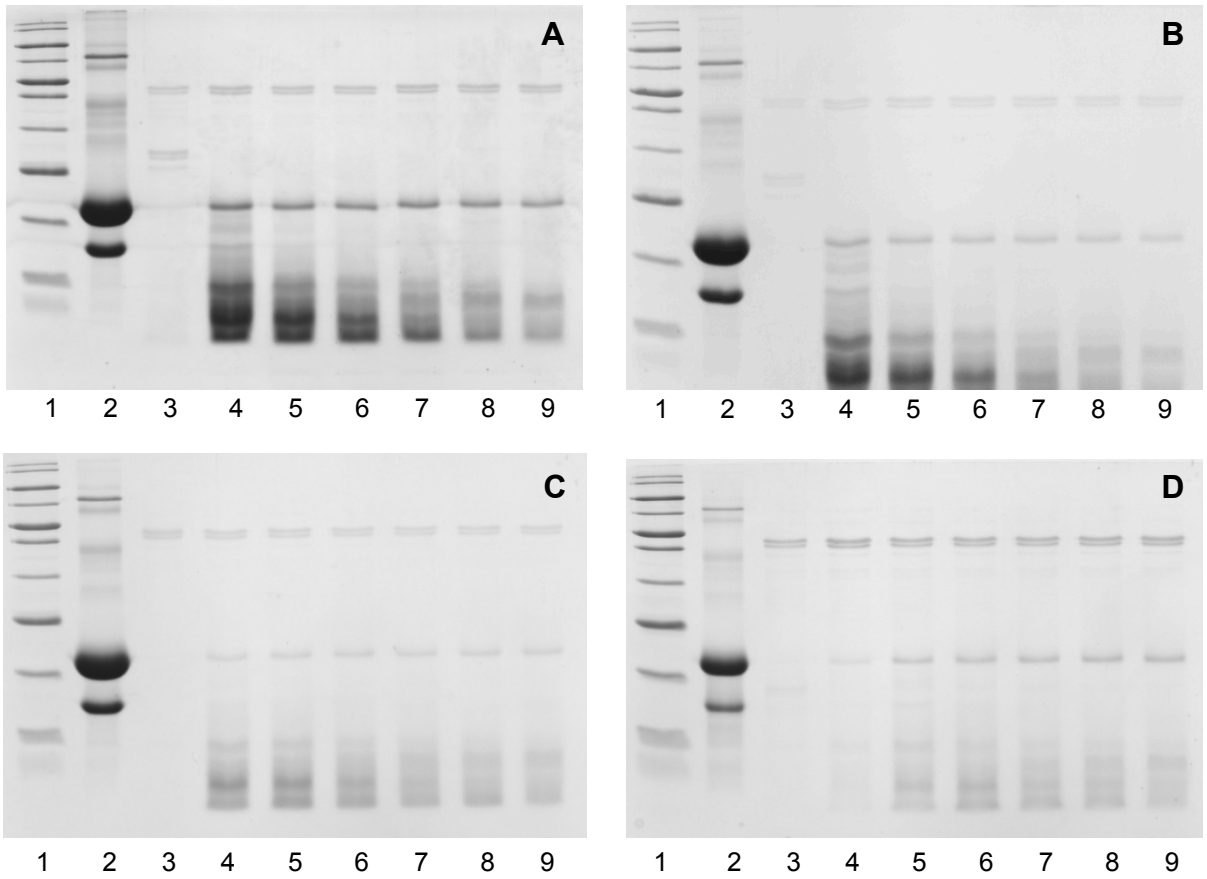


Figure 7.

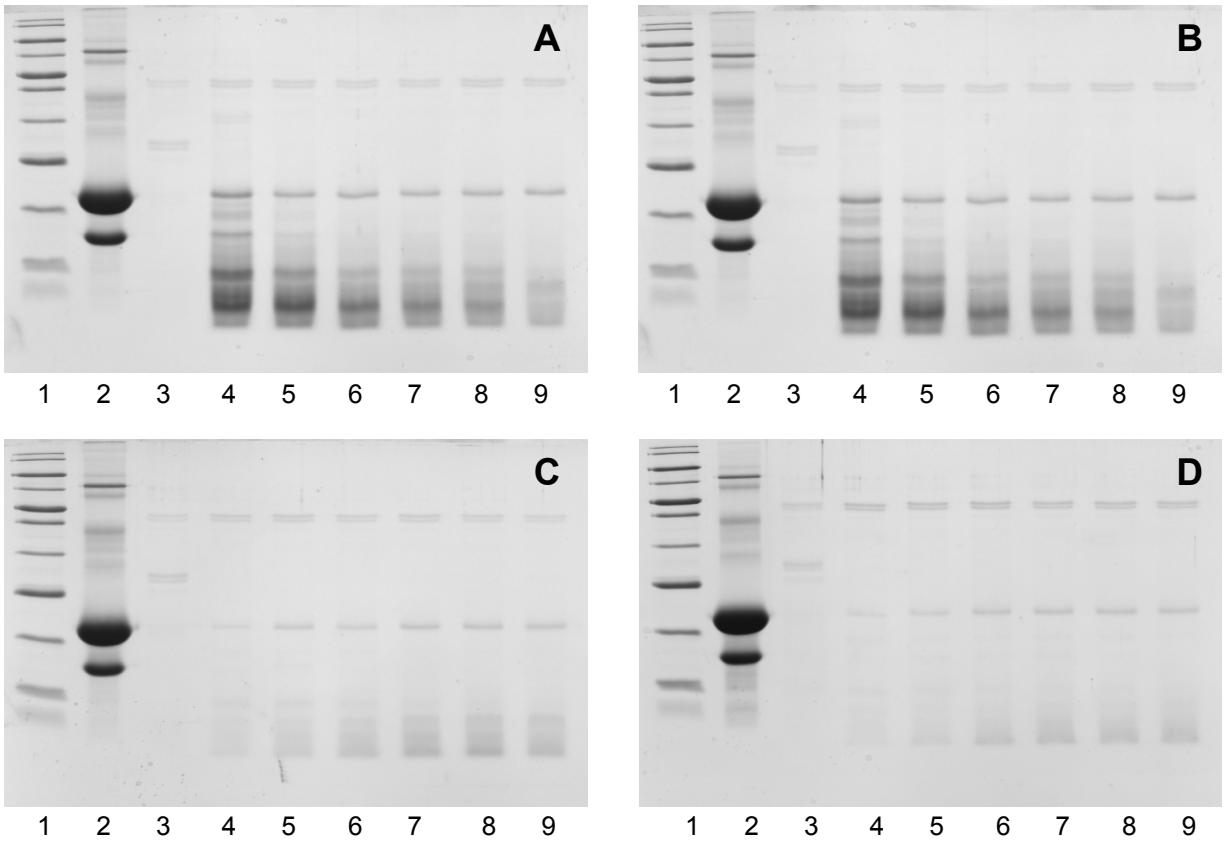


Figure 8.