



Effect of initial protein concentration and pH on *in vitro* gastric digestion of heated whey proteins



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ABSTRACT

The *in vitro* digestion of heated whey protein aggregates having different structure and physicochemical properties was evaluated under simulated gastric conditions. Aggregates were formed by heating whey protein isolates (WPI) at 3–9% w/w initial protein concentration and pH 3.0–7.0. Results showed that high protein concentration led to formation of larger WPI aggregates with fewer remaining monomers. Aggregates formed at high protein concentrations showed slower degradation rate compared to those formed at low protein concentration. The effect of initial protein concentration on peptide release pattern was not apparent. Heating pH was a significant factor affecting digestion pattern. At pH above the isoelectric point, the majority of the proteins involved in the aggregation, and aggregates formed at pH 6.0 were more susceptible to pepsin digestion than at pH 7.0. At acidic conditions, only small amount of proteins was involved in the aggregation and heated aggregates were easily digested by pepsin, while the remaining unaggregated proteins were very resistant to gastric digestion. The potential physiological implication of these results on satiety was discussed.

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1. Introduction

Microstructure and physicochemical properties of foods have significant effects on the digestibility due to the influence on susceptibility to enzyme reaction in the gastrointestinal (GI) tract. A fundamental understanding of the relationship between microstructure of food matrix and its performance during human GI digestion can assist food manufacturers in developing the next generation of structured food for health (Kong & Singh, 2008). Modification of food microstructure could reduce the allergenic potential by enhancing the accessibility of the cleavage site of digestive enzymes on allergenic substances (Sathe & Sharma, 2009; Thomas et al., 2007; Wickham, Faulks, & Mills, 2009). It has been shown that conformational changes of some food allergens caused by preheating could increase the allergen digestibility and therefore reduce the allergic potential (Cabanillas et al., 2011; Tong et al., 2012). On the other hand, the design of food microstructure that allows controlled release of bioactive ingredients from food through the GI tract is essential to maximise the delivery efficiency.

Food proteins usually experience significant changes as they pass through the GI tract, and the digestibility of proteins is signif-

icantly influenced by its structure. Native β -lactoglobulin (β -lg) is very resistant to pepsin and chymotrypsin digestion, while disruption of native structure and conformation by thermal treatment exposes susceptible peptide bonds and drastically decreases the resistance to proteolytic digestion (O'Loughlin, Murray, Kelly, Fitzgerald, & Brodtkorb, 2012). Several studies found that the digestibility of interfacial proteins at oil–water or air–water interface could be controlled by manipulating the interfacial unfolding (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; MalakiNik, Wright, & Corredig, 2010; Sarkar, Goh, Singh, & Singh, 2009). Static high pressure has been shown to increase susceptibility to pepsin digestion due to the unfolding of protein under high pressure treatment (Chicón, Belloque, Alonso, & López-Fandiño, 2008; Zeece, Huppertz, & Kelly, 2008). Except for unfolding of protein, it has been recently found that the aggregate size and porosity might also play a role in promoting susceptibility to enzymatic hydrolysis (O'Loughlin et al., 2012).

The effect of heat treatment on the digestibility of whey protein *in vitro* and *in vivo* has been well documented, and studies have compared the digestibility of heat denatured protein to native protein (Peram, Loveday, Ye, & Singh, 2012; Takagi, Teshima, Okunuki, & Sawada, 2003). However, little has been known on the relationship between size, shape and structure of whey protein aggregates and their intragastric behaviour. Thermal processing of whey protein leads to the formation of aggregates having various sizes and shapes depending on several factors, such as protein concentration, pH, salt concentration and heating temperature. The average size

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of the aggregates increases with increasing protein concentrations when heated at neutral pH. It has been reported that heating β -lg at increasing pH from 2.0 to 5.8 to 7.0 resulted in rod-like, spherical, and worm-like primary aggregates, respectively (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008). These aggregates have been shown not only to differ in sizes and morphologies, but also in their internal structures and fractal dimensions. It is highly possible that various structured whey protein aggregate formation would be able to modulate the susceptibility of whey protein to proteolysis.

Most studies on *in vitro* gastric digestibility of whey proteins are related to either their allergenicity or the released bioactive peptide profile. Much less focus has been on its relation to satiety. Growing evidences show that high protein diets increase greater satiety when compared to isoenergetic carbohydrate or fat (Halton & Hu, 2004; Veldhorst et al., 2008). Whey proteins have been suggested to be superior to other proteins in promoting satiety on both short-term and intermediate-term food intake (Anderson, Tecimer, Shah, & Zafar, 2004; Luhovyy, Akhavan, & Anderson, 2007; Yu, South, & Huang, 2009). However, the mechanisms of satiety property of whey proteins and their contribution to food intake regulation have not been fully defined. It is recognised that structures and physicochemical properties of foods play important roles in determining satiety. A better understanding of how protein structures relate to their degradation properties under gastric conditions might help to provide complementary information of gastric emptying and thus the influence on satiety in the GI tract. Studies using *in vitro* stomach model have shown that food emulsions stabilised by different emulsifier led to different stomach behaviours due to the flocculation and coalescence occurred during gastric digestion, which might lead to different stomach emptying rate and feelings of fullness (van Aken, Bomhof, Zoet, Verbeek, & Oosterveld, 2011). Addition of acid-unstable emulsions in preprocessed foods led to rapid gastric emptying, while acid-stable emulsions delayed gastric emptying and maximise satiety signaling (Marciani et al., 2007). The stability in acidic stomach environment may lead to a prolonged feeling of fullness and early satiety during the meal.

Accordingly, the objective of this study is to investigate the effect of different aggregate formation on *in vitro* gastric digestibility of whey protein. The possible physiological relevance of *in vitro* intragastric behaviours to stomach emptying and satiety will also be discussed. A range of protein concentrations (3–9%) and pH conditions (pH 3.0–7.0) were chosen to form whey protein aggregates with different size, shape or structure. Reducing and non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE, and dynamic light scattering (DLS) are combined to evaluate the *in vitro* digestibility of whey protein aggregates.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was kindly donated by Davisco Food International (BiPro, Le Sueur, MN). The WPI constituted of 95.4% total solid, 93.1% protein, and 2.1% ash. The ion composition of WPI is as follows (% w/w): $\text{Fe}^{2+} = 0.005$, $\text{Ca}^{2+} = 0.13$, $\text{Mg}^{2+} = 0.025$, $\text{K}^+ = 0.075$. Pepsin was obtained from Sigma–Aldrich (St. Louis, MO). Unless otherwise stated, all of the chemicals used were of analytical grade.

2.2. Heat treatment of WPI

Whey protein stock solution (10% w/w) was prepared by dissolving WPI in Millipore water (18.2 M Ω) with continuous stirring for 2 h, and stored overnight at 4 °C for complete hydration. WPI stock

solution was diluted to 3%, 5%, 7%, and 9% w/w and the pH was adjusted to 7.0 using 1 N and 0.1 N NaOH before heat treatment. For 5% WPI solutions, the pH were also adjusted to 3.0, 4.0, and 6.0 using 1 N and 0.1 N HCl. The samples were heated at 85 °C for 30 min in a temperature controlled water bath, cooled by running tap water, and stored at 4 °C before the *in vitro* digestion.

2.3. *In vitro* pepsin digestion

All the preheated WPI solutions were diluted to 3% w/w in Millipore water before enzymatic hydrolysis. The simulated gastric fluid (SGF) consisted of 0.034 M NaCl and the pH was adjusted to 1.2 using HCl. Pepsin solution was prepared freshly for each assay by dissolving pepsin in SGF by vortexing several times over a period of 5 min and the resulting solution was placed on ice. The *in vitro* gastric model consisted of a conical flask (50 mL) containing 5 mL of SGF-pepsin maintained at 37 °C with continuous shaking at 95 rpm/min in a temperature-controlled water bath. The SGF-pepsin solution was pre-incubated for 5 min, followed by addition of 5 mL of diluted WPI solutions. The ratio of pepsin to WPI was 1:250 on a weight basis. Aliquots (100 μ L) were withdrawn into Eppendorf vials containing 70 μ L NaOH (0.1 M) to inactivate the enzyme after 0, 0.5, 2, 5, 10, 20, 30, 60, 120 min of incubation.

2.4. Electrophoresis

SDS–PAGE was carried out using a modification of Laemmli method under both reducing and nonreducing conditions. Protein samples were diluted to 1.5 g/L in 0.5 M Tris–HCl buffer (pH 6.8) before being solubilised in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). Sample buffer for reducing conditions also contained 5% β -mercaptoethanol. For native-PAGE, samples were solubilised with native sample buffer (Bio-Rad Laboratories).

Gels (15% acrylamide for resolving gel and 4% acrylamide for stacking gel) were run in a mini Protein II electrophoresis system (Bio-Rad Laboratories) using an electrode stock buffer at a voltage of 120 V. Proteins were stained with Coomassie brilliant blue R250 in an acetic acid:isopropanol:H₂O staining solution (3:10:17 by volume), and destained in an acetic acid:methanol:H₂O solution (1:4:5 by volume). Molecular weight was determined by comparison to a standard (PageRuler unstained broad range protein ladder: Thermo Scientific, Rockford, IL). Imaging was accomplished with Alphamager system (Alpha Innotech Corporation, Santa Clara, CA).

2.5. DLS measurement

The average particle diameter of WPI aggregates before and during *in vitro* digestion was measured by DLS using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) equipped with 633 nm laser and 173° detection optics. Each sample was measured by dilution with 0.5 M Tris–HCl (pH 6.8) buffer to a final concentration of 0.1%. During the measurement, the laser light was directed and focused on the cuvette with 1.2 mL sample solutions. For each sample, three measurements were conducted with at least 12 runs and each run lasted for 10 s. All experiments were replicated at least twice. The intensity measured by DLS was used to indicate the total number of the aggregates in the sample.

3. Results and discussion

3.1. Effect of initial protein concentration on digestion

3.1.1. Electrophoresis

It has been well documented that varying initial protein concentrations during heating results in formation of aggregates

having different sizes as shown by changes in intrinsic viscosity (Vardhanabhuti & Foegeding, 1999). In this study, WPI aggregates were formed at WPI concentrations ranging from 3% to 9% (w/w) at pH 7.0. The *in vitro* gastric digestion patterns of WPI aggregates were examined using native-PAGE and SDS-PAGE under both non-reducing and reducing conditions, which allowed the determination of the dissociation of the aggregates during pepsin digestion as well as the nature of the intermolecular interactions involved in the aggregation process.

Native-PAGE shows two types of aggregates formed after WPI was heated at 85 °C for 30 min (Fig. 1, lane 2). Band labeled “Aggregates 1” represents very large aggregates that could not enter the stacking gel, while “Aggregates 2” represents those that could enter the stacking gel but not the resolving gel. These two types of aggregates could differ in size, shape and/or charge. Peram et al. (2012) also observed two types of large aggregates that did not enter the resolving gel and stacking gel, respectively. For 3% WPI, the decrease in the intensity of native β -lg and corresponding increase in dimers, trimers, oligomers and aggregates indicated that only a small quantity of β -lg monomers remained in the native state,

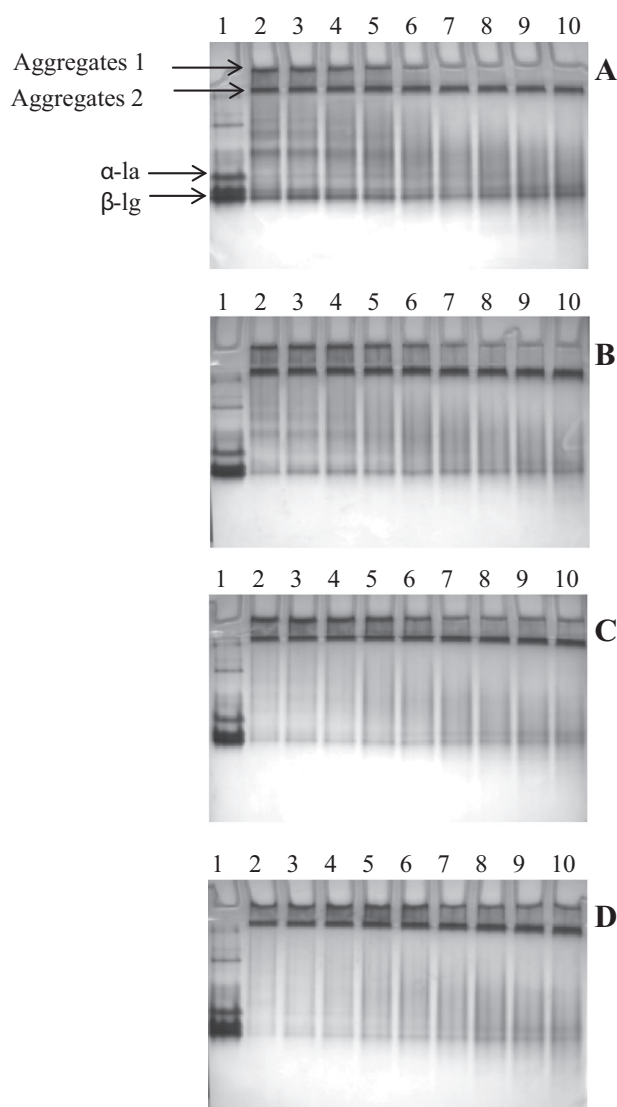


Fig. 1. Native-PAGE profiles of *in vitro* digestion of WPI heated at different initial protein concentrations: (A) 3%; (B) 5%; (C) 7%; (D) 9%. Lane 1, unheated WPI; lane 2, preheated WPI; lane 3–10, samples digested for 0.5, 2, 5, 10, 20, 30, 60, and 120 min, respectively.

and the majority are involved in the aggregation after heating (Fig. 1A) similar to heated β -lg as previously reported by Peram et al. (2012). With increasing WPI concentrations, the amount of monomers, dimers and oligomers decreased (Fig. 1A to D, lane 2), denoting higher extent of aggregation and lower amount of native proteins. During digestion, native-PAGE shows decreasing Aggregates 1, increasing Aggregates 2, and decreasing dimer and oligomer (Fig. 1, lane 2–10). The degradation rate of aggregates was greatly influenced by initial protein concentration. Aggregates 1 of 3% and 5% WPI were completely digested by pepsin within 30 min (Fig. 1A and B), while those of 7% and 9% WPI were not fully digested even after 120 min (Fig. 1C and D). Aggregates 2 remained almost constant for 3% WPI during 2 h digestion but increased slightly in higher protein samples which was likely due to digestion of Aggregates 1. The dimers, trimers, and oligomers presented in 3% WPI were easily digested within 5 min. Interestingly, β -lg and α -la bands did not change much during digestion and higher protein samples showed much less intense monomer bands. Since native-PAGE revealed the actual compositions of the samples (e.g., no bond breaking), the results indicated that the majority of the digested samples was composed of the aggregates and peptides and only small amount of native monomers.

Compared to native-PAGE, non-reducing SDS-PAGE showed a noticeable weaker intensity of the bands for both types of aggregates and an increase in the intensity of monomer and oligomer bands in undigested 3% heated samples (Supplementary Fig. 1A, lane 2), while all aggregates were broken into monomers in reducing SDS-PAGE (Fig. 2, lane 3). As expected, these results confirm that although disulphide bonds contributed to the aggregation at pH 7.0, noncovalent interactions were also involved. As protein concentration increased, non-reducing PAGE showed less intense aggregate as well as monomer bands (Supplementary Fig. 1A–D, lane 2), indicating higher contribution of covalent bonds in WPI aggregates formed at higher concentrations.

In-vitro digestion analysis by non-reducing SDS-PAGE (Supplementary Fig. 1) showed similar trend of degradation for Aggregates 1. Aggregates formed at lower protein concentrations were digested at a faster rate compared to aggregates formed at higher protein concentrations. Presented as faint band before digestion, Aggregates 2 significantly increased after 5 min digestion. This was due to the dissociation of noncovalent bonds of Aggregates 1, resulting in smaller disulphide linked aggregates and peptides. The dimers and oligomers presented in 3% and 5% heated WPI were digested almost completely within 5 min, similar to what shown in the native-PAGE. The intensity of the monomers decreased in the first 5 min of digestion and remained almost constant thereafter.

A notable difference observed in reducing SDS-PAGE (Fig. 2) among four samples is the degradation of β -lg. The lower the protein concentration used in preheating, the more β -lg presented in the 2 h digested samples. There were much more β -lg monomers presented in 3% WPI than 9% WPI after 2 h digestion. Combining the results obtained from all PAGE analysis, it is obvious that monomers presented in reducing SDS-PAGE included both native β -lg and nonnative β -lg involved in the aggregation but further dissociated by reducing agent in SDS-PAGE sample buffer. Since native β -lg is very resistant to pepsin digestion, the decrease in the intensity of β -lg band was due to the degradation of nonnative β -lg, and β -lg band presented after 2 h digestion was mainly composed of native monomers. Compared to 3% sample, dissociation of the aggregates of 9% digested sample showed much less intense β -lg band, indicating that digested aggregates of 9% sample were composed of peptides linked by disulphide bonds. Dissociation of these digested aggregates resulted in increased peptides but not native β -lg. It should be noted that digestion pattern of α -la among 3–9% protein samples was not different. Increasing digestion time resulted in less intense α -la band.

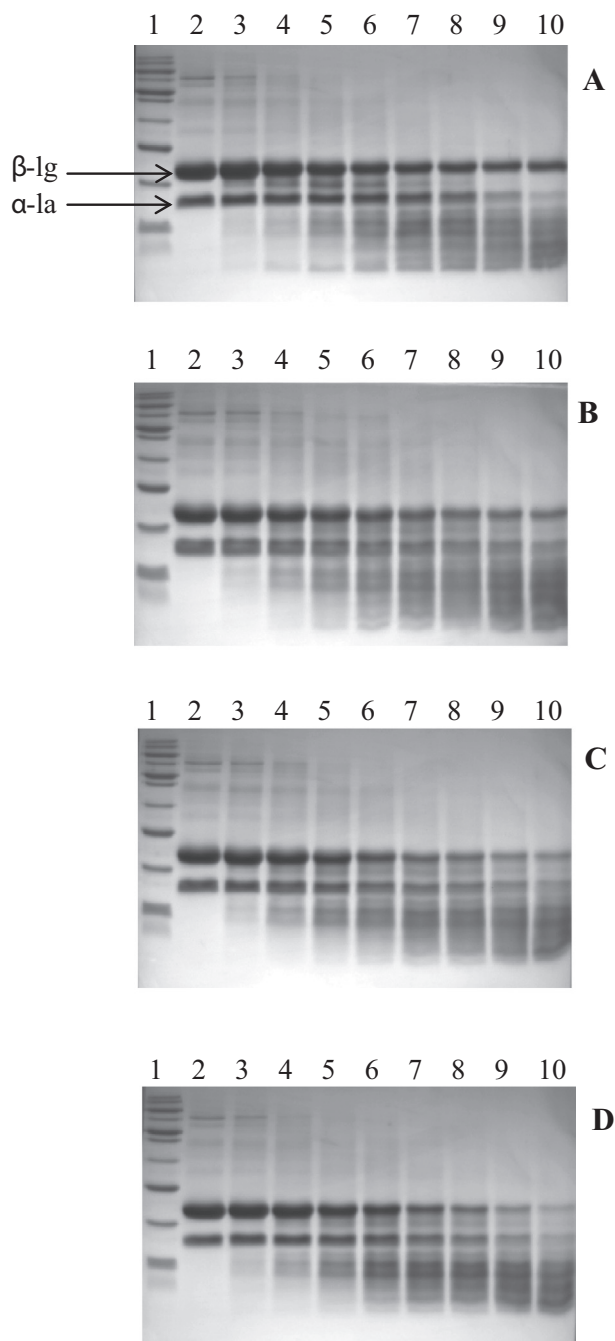


Fig. 2. SDS-PAGE profiles of *in vitro* digestion of WPI heated at different concentrations: (A) 3%; (B) 5%; (C) 7%; (D) 9%. Lane 1, maker; lane 2, preheated WPI; lane 3–10, samples digested for 0.5, 2, 5, 10, 20, 30, 60, and 120 min, respectively.

Peptides (appearing as bands below α -la band) were observed right after 0.5 min digestion. The amount of peptides increased with prolonged digestion time. Compared to peptides detected in the reducing SDS-PAGE, there was smaller quantity of peptides in the non-reducing SDS-PAGE as indicated by the less intense and narrower bands below β -lg band. This confirmed that digestion resulted in peptides that were held by both covalent and non-covalent bonds. In several studies investigated the *in vitro* digestion of β -lg, no peptide band could be detected after about 30 min digestion because of the further cleavages of the original peptides by pepsin (Bateman, Ye, & Singh, 2010; Peram et al., 2012). Different results found in this study could be due to the different pepsin:protein ratio used.

3.1.2. Dynamic light scattering

DLS could provide complementary information of aggregate degradation during *in vitro* digestion. The results obtained from electrophoresis indicated the nature of the intermolecular interactions involved in the aggregation, as well as their effect on the dissociation of the aggregates during *in vitro* digestion; however, a large number of peptides in the sample were too small to be detected. More specific information of changes of aggregates could be obtained by DLS. The changes in particle sizes and their distribution obtained by DLS allowed the investigation of the degradation rate of the aggregates.

The Z-average diameters of heated aggregates formed by 3%, 5%, 7%, and 9% WPI were 39.0, 43.3, 47.3, and 67.7 nm, respectively. The aggregate size decreased gradually during the first 10 min digestion, with the most remarkable reduction observed for 9% WPI which showed a drastic reduction from 67.7 to 40.7 nm within 10 min (Fig. 3A). For all four samples, the average aggregate size remained almost constant after 30 min digestion, suggesting that the aggregates could not be completely digested within 2 h, which confirmed the results obtained in the native-PAGE.

DLS intensity (counts/s) has been used to indicate the concentration of the particles (Dai, Liu, Coutts, Austin, & Huo, 2008; Jans, Liu, Austin, Maes, & Huo, 2009; Liu & Huo, 2009). In this study, in order to compare the total number of different sized particles, the intensity was normalised according to Rayleigh's approximation. Since large particles scatter much more light than small particles, the intensity of scattering of a particle is proportional to the sixth

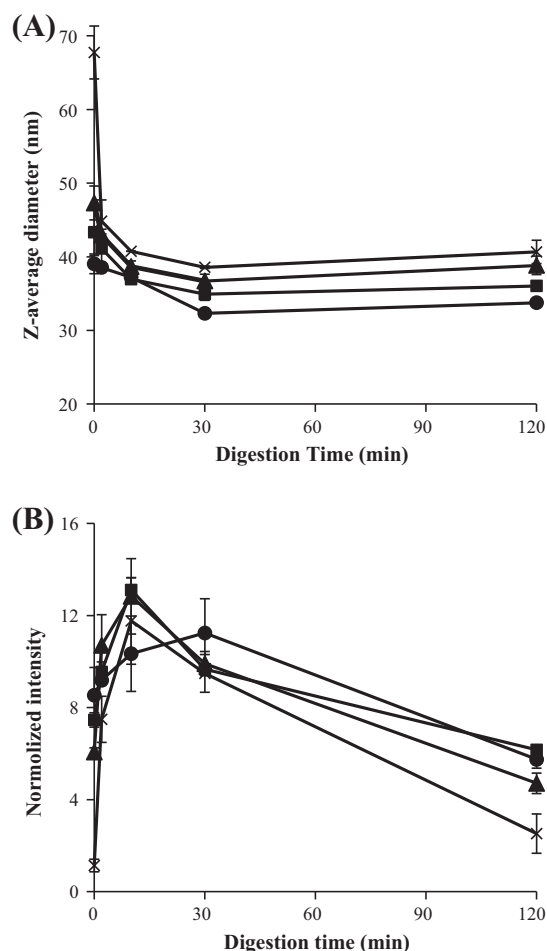


Fig. 3. Particle size (A) and normalised DLS intensity (B) of WPI aggregates heated at pH 7 during *in vitro* gastric digestion: ●: 3%; ■: 5%; ▲: 7%; ×: 9%.

power of its diameter. Hence, the total intensity measured by DLS was divided by the sixth power of its diameter to indicate the number of the aggregates presented in the sample. As shown in Fig. 3B, the higher the protein concentration, the fewer the number of the aggregates. The average diameter of 3% aggregates was only half of that of 9% aggregates, while the total number of 3% aggregates is eight times higher than 9% aggregates. During digestion, the number of the aggregates slightly increased within 30 min for 3% and 10 min for 5–9%, but gradually decreased afterwards. This was because large aggregates were disintegrated into smaller ones (as shown in Fig. 1) which were then disrupted by pepsin into peptides. After 2 h of digestion, the number of the aggregates was reduced by 31.9%, 23.2%, and 22.1% for 3%, 5%, and 7% of WPI, respectively, but the number of 9% aggregates increased by 18.3%, indicating that larger aggregates tended to have slower degradation rate.

Heat-induced aggregation of WPI reduces tertiary and quaternary structures of protein, and exposes previously buried hydrolytic cleavage sites, resulting in a marked increase in the number of peptide bonds available for enzymatic cleavage (O'Loughlin et al., 2012). Heating WPI at different concentrations leads to the formation of aggregates with different sizes. From the results obtained in this study, it can be presumed that when digested at the same pepsin:protein ratio larger aggregates have smaller total surface area, which provides fewer cleavage sites for enzyme reaction than smaller aggregates. Hence, the smaller aggregates have higher degradation rate.

3.2. Effect of pH on digestion

3.2.1. Electrophoresis

The aggregation of WPI has been shown to be very sensitive to pH (M. A. M. Hoffmann & van Mil, 1999). In this study, WPI was heated at 5% and pH 3.0, 4.0, 6.0, and 7.0, and their digestion patterns were monitored by electrophoresis and DLS. As shown in native-PAGE (Fig. 4), WPI was very stable under acidic conditions during heating. The majority of β -lg and α -la remained in their native states and only very small amount of Aggregates 1 and 2 was observed at pH 3.0. Increasing pH to 4.0 promoted the aggregation since the pH was getting closer to pI and the repulsions between molecules was reduced. Increasing pH further to above the pI of the protein induced much higher extent of denaturation and aggregation as shown by reduced intensity of monomer bands and more intense aggregate bands. Aggregates formed at pH 6.0 were larger than those at pH 7.0 such that most could not enter the stacking gel. The formation of larger aggregates at pH 6.0 is expected. Aggregates 1 formed at pH 6.0 and 7.0 were completely digested within 60 and 30 min, respectively, but Aggregates 2 could not be fully digested within 120 min (Fig. 4). There were only small amount of Aggregates 2 present in undigested samples at pH 6.0, but more was present after 10 min digestion, probably due to the degradation of Aggregates 1, while the amount of Aggregates 2 of pH 7.0 seemed constant during 2 h digestion. Digestion behaviour of aggregates formed at pH lower than pI was significantly different from those formed at pH above pI. Aggregates 1 formed at pH 4.0 were easily broken down within 5 min digestion, and Aggregates 2 were fully digested within 120 min. Surprisingly, Aggregates 2 formed at pH 3.0 were more resistant to pepsin digestion than at pH 4.0. For both pH 3.0 and 4.0, the β -lg monomers that were not involved in the aggregation remained almost constant during 2 h digestion. However, α -la monomers were more sensitive to pepsin since no α -la monomers were detected after 30 min for either pH 3.0 or pH 4.0. Since the majority of the proteins at pH 3.0 and 4.0 remained in their native states, much less number of peptides was formed at these pH values compared to those at high pH (Fig. 5).

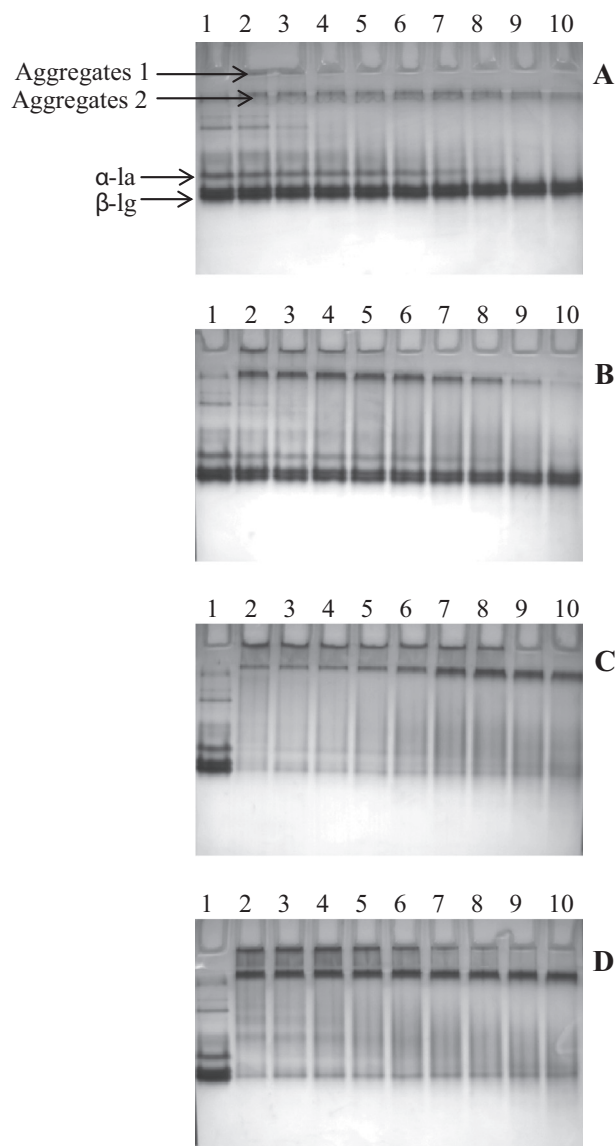


Fig. 4. Native-PAGE profiles of *in vitro* digestion of 5% WPI preheated at different pH: (A) pH 3.0; (B) pH 4.0; (C) pH 6.0; (D) pH 7.0. Lane 1, unheated WPI; lane 2, preheated WPI; lane 3–10, samples digested for 0.5, 2, 5, 10, 20, 30, 60, and 120 min, respectively.

Since noncovalent bonds were disrupted completely, the aggregates shown on non-reducing SDS-PAGE were only cross-linked by disulphide bonds. As shown in native-PAGE that a large amount of aggregates were still present after 120 min digestion in samples at pH 7.0, non-reducing SDS-PAGE indicated that the undigested aggregates were mainly linked by covalent bonds. Furthermore, the amount of Aggregates 2 for both pH 6.0 and pH 7.0 increased with digestion within 30 min as shown on non-reducing SDS-PAGE (Supplementary Fig. 2), indicating that noncovalently linked aggregates were easier to be digested than disulphide bonded aggregate. The amount of Aggregates 2 of pH 6.0 then decreased from 30 to 120 min, while there was only a slight decrease of aggregates of pH 7.0. It appears that pH 7.0 has more α -la remain undigested than pH 6.0 (Fig. 5), probably due the different structured aggregates formation at these pH. It has been reported that similar reorganised structure was formed when α -la was heated at 80 °C at either pH 6.0 or 7.0, while the reorganised structure was less well-defined after heating at 90 °C at pH 6.0 due to the alterations of the disulphide bonds (Fang & Dalgleish, 1998). The heating tem-

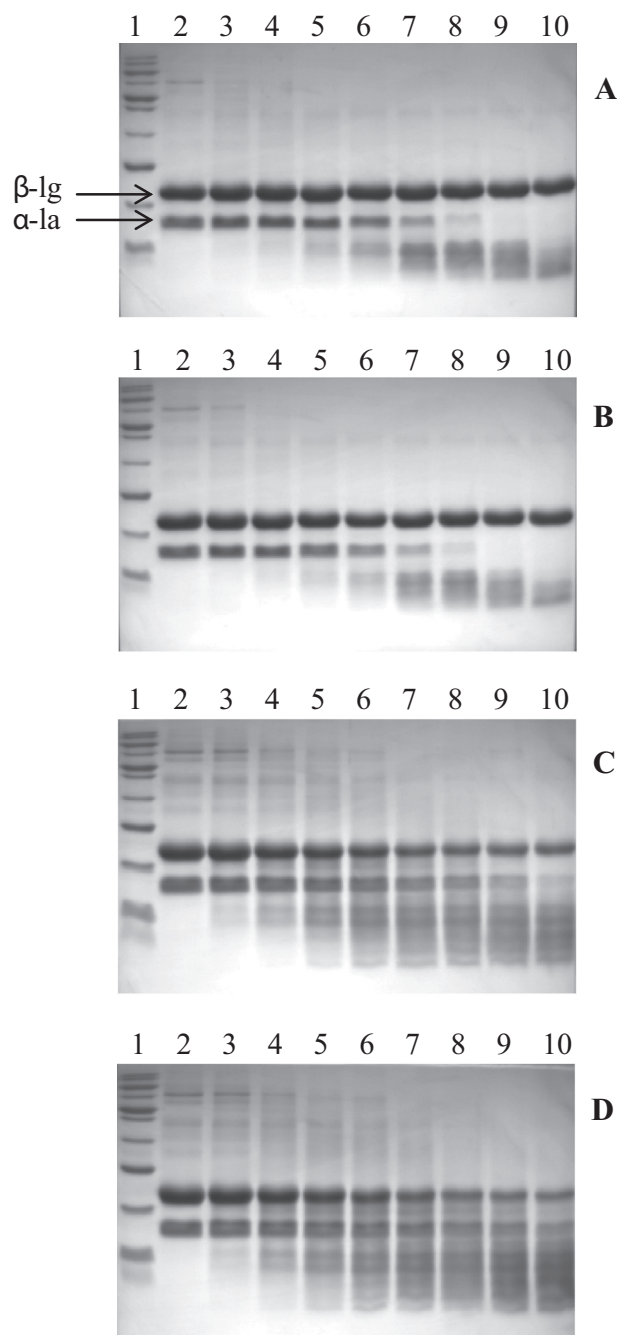


Fig. 5. SDS-PAGE profiles of *in vitro* digestion of 5% WPI preheated at different pH: (A) pH 3.0; (B) pH 4.0; (C) pH 6.0; (D) pH 7.0. Lane 1, marker; lane 2, preheated WPI; lane 3–10, samples digested for 0.5, 2, 5, 10, 20, 30, 60, and 120 min, respectively.

perature used in this study is 85 °C, though the mechanism is not clear, it is possible that different aggregate structures were formed at pH 6.0 and pH 7.0, which could have different resistance to pepsin. The peptide patterns between pH 6.0 and 7.0 appeared similar though a slightly more intense bands were observed at pH 7.0. All PAGE results confirmed that β -Ig in heated samples at low pH was present mostly in native form which was highly resistant to pepsin. The amount of peptides in low pH samples was much lower than high pH samples.

3.2.2. Dynamic light scattering

In the last section, the changes of number of aggregates obtained from DLS were used to indicate the degradation rate of

the aggregates since all of the samples only have single peak. However, during the digestion of aggregates formed at pH 4.0 and 6.0, more than one peak were observed during digestion from particle size measurement. Hence, it is hard to calculate the number of the aggregates; instead the particle size distribution would be more helpful to monitor the changes of aggregate size during digestion. Volume distribution was used since multiple peaks were found in some samples. Particle size distribution (Fig. 6) only shows a single peak between 1 and 10 nm for samples heated at pH 3.0, representing the native WPI monomers. No changes in particle size distribution were observed through 120 min digestion, confirming the resistance of β -Ig to pepsin digestion. For WPI heated at pH 4.0, large aggregates ($D = 223$ nm) were formed, but were broken down to much smaller ones ($D = 125$ nm) within 2 min digestion. The particle size distribution with single peak maintained between 1 and 10 nm through 30–120 min digestion, indicating no aggregates presented in the sample after 30 min, which was consistent with the results obtained from electrophoresis. Unlike heating at neutral pH, where protein undergo irreversible thermal denaturation, heating β -Ig at pH lower than pI only induced limited disulphide-mediated polymerisation. It is known that unheated β -Ig is very resistant to pepsin digestion at typical gastric pH (1–3). As a result, the heat stability of β -Ig under acidic conditions is responsible for the poor accessibility to pepsin digestion.

The average diameter for aggregates formed at pH 7.0 was 43.3 nm. During 120 min digestion, only a slight reduction of the size was observed after 10 min digestion, and the size maintained unchanged afterwards. For WPI heated at pH 6.0, much larger aggregates with average diameter equals to 212 nm were formed. The size remained constant within the first 10 min digestion, but decreased drastically to 36.8 nm after 30 min digestion. After 120 min, the particle size distribution shows two peaks with average diameters of 39.0 and 4.79 nm.

Heat-induced aggregation of WPI is pH dependent. Aggregation via chemical reactions increased when increasing pH from near pI to above pI due to the increased reactivity of thiol groups (Hoffmann & van Mil, 1997). Formation of physical bonds between proteins, on the other hand, is enhanced at pH values close to the pI of the protein because of reduced intermolecular repulsion (Verheul et al., 1998). The nature of the intermolecular interactions involved in the aggregation could play an important role in the degradation rate of WPI aggregates under gastric digestions. It has been reported that pepsin has a hydrophobic binding site that prefers to attack a peptide linkage which contains an aromatic amino-acid (Tang, 1963). It is possible that heating WPI at pH 6.0 resulted in more hydrophobic interactions, which might play an important role in improving the susceptibility to pepsin digestion than WPI heated at pH 7.0.

3.3. Possible physiological implication in satiety

The pepsin:protein ratio used in *in vitro* studies varied from 11:1 to 1:750 depending on the purpose of the study (Astwood, Leach, & Fuchs, 1996; Hur, Lim, Decker, & McClements, 2011; Zece et al., 2008). The digestibility of the protein was strongly affected by the pepsin:protein ratio. In order to better simulate the gastric conditions in human GI tract, it is necessary to use the appropriate pepsin:protein ratio. It has been reported that the daily pepsin secretion in adults is 20–30 kU of enzyme activity at 37 °C, while a typical adult dietary intake of protein comprises around 75 g/24 h, giving a pepsin:protein ratio of 1:750 if consider the enzyme activity of pepsin is around 250 U (Hur et al., 2011). Moreover, subjects in clinical trials studying the satiety effect are usually asked to drink the whey protein beverage as quick as possible within 15 min, or even within 3 min (Burton-Freeman, 2008; Morifuji et al., 2010; Pal & Ellis, 2010; Poppitt et al., 2011). Considering that

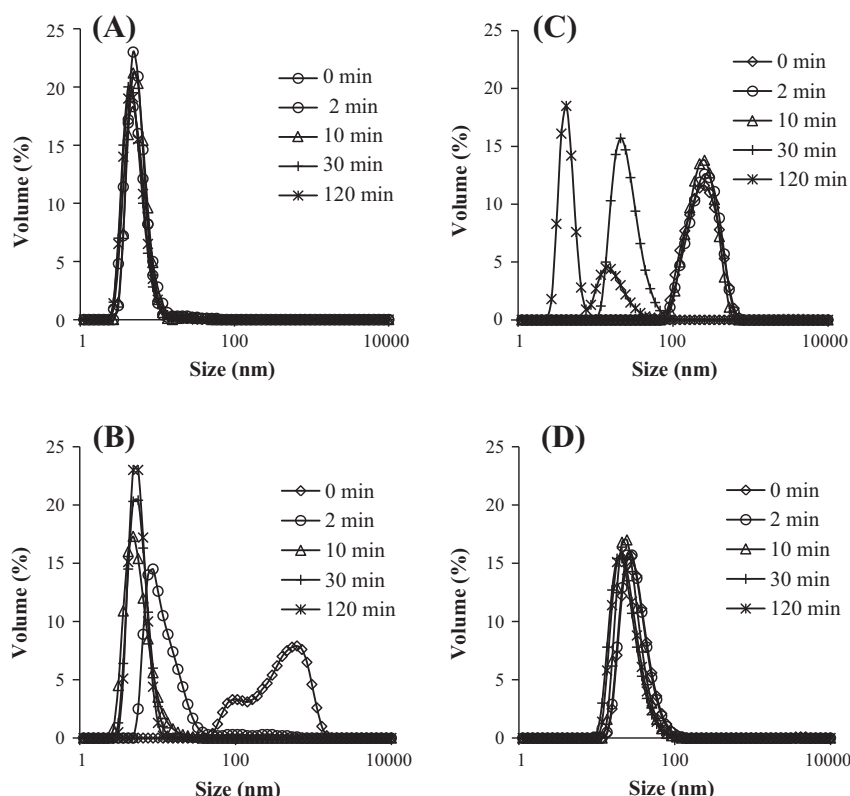


Fig. 6. Particle size distributions of WPI aggregates at selected times during *in vitro* gastric digestion of 5% w/w WPI heated at (A) pH 3.0; (B) pH 4.0; (C) pH 6.0; (D) pH 7.0.

increase in protein content in meal increases the secretion of pepsin, the pepsin:protein ratio was chosen at 1:250 in this study.

Although the mechanism of the satiety effect of whey protein has not been fully defined, it has been suggested that in general the gastric emptying rate plays an important role in short-term food intake. Foods with controlled degradation rate in stomach might slow the gastric emptying, thus enhance the feeling of fullness and lead to earlier meal termination (Norton, Moore, & Fryer, 2007). In addition, the rate of nutrient release in the small intestine also influences food intake regulation. Satiety of whey proteins has been suggested to be related to the release of amino acids and bioactive peptides (Luhovyy et al., 2007; Veldhorst et al., 2008). Although fast digested protein does not show benefit in slowing stomach emptying, it causes elevated level of plasma amino acids, which is associated with a loss of appetite (Westerterp-Plantenga, 2003). It has been suggested that the bioactive peptides released after ingestion of protein have the potential to exert their actions through gut satiety mechanisms (Luhovyy et al., 2007). In a study comparing the digestibility of native and heated whey protein *in vitro* and *in vivo*, it has been found that the rate of body weight increase in rats fed heated whey protein was higher than that of rats fed unheated whey protein due to the easy digestion and quick absorption of heated whey protein (Kitabatake & Kinekawa, 1998). In another animal study investigating the effect of heat treatment and the gelation of milk protein on digestion using female adult minipigs, the authors found that caseins and β -lg, respectively, were sensitive and resistant to hydrolysis in the stomach with the unheated matrices, but showed similar digestion with the heated matrices (Barbé et al., 2012). However, in most clinical trials investigating satiety effect of whey protein, not much detailed information about sample preparation is available. For example, in a double blind cross-over study, 500 mL protein-enriched beverage preloads containing 1%, 2% and 4% (w/w) whey protein significantly reduced hunger but the effect was short term and did not

significantly impact subsequent food intake (Poppitt et al., 2011). Although the authors stated that the preloads were matched with volume, flavour and sweetness to mask the difference in protein content, the pretreatment of the whey protein beverage is unclear. The results obtained in this study suggested that sample preparation and the physico-chemical properties such as size and shape of whey proteins influenced by heat treatment and heating conditions clearly lead to different degradation behaviour in the stomach. This could lead to differences in rate of gastric emptying, the feeling of fullness, and eventually satiety and subsequent food intake. Therefore, more studies are necessary to understand the profile of amino acid and peptide release of whey protein aggregates having different structures. Clinical studies are needed to confirm the effect of amino acid and peptide release on satiety and subsequent food intake.

4. Conclusion

In this study, whey protein aggregates were formed by heating at different protein concentration and pH, resulting in the formation of aggregates having different physico-chemical properties. *In vitro* digestion patterns of these aggregates were investigated. At neutral pH, WPI heated at high protein concentration resulted in the formation of large aggregates with higher contribution from covalent bonds. Aggregates formed at higher initial protein concentration had slower degradation rate compared to those formed at low protein concentrations. Heating pH was found to be a significant factor affecting digestion behaviour. Heated WPI formed at pH above pI were more susceptible to gastric digestion than those formed at pH below pI because the unfolding and aggregation undergone at near neutral pH exposed more hydrophobic residue and accessible peptide bond for enzyme reaction. When the pH decreased to below the pI, proteins were more resistant to

aggregation as well as digestion. Only small amount of WPI was involved in the aggregation at pH 4.0, and the heated aggregates were completely digested within 30 min. At pH 3.0, the majority of the protein did not form aggregates during heating, which made them very resistant to gastric digestion, resulting in lower amount of released peptides. These results indicate that structure and physico-chemical properties of protein aggregates significantly affect their digestion behaviour which could potential result in differences in their effect on satiety and energy intake.

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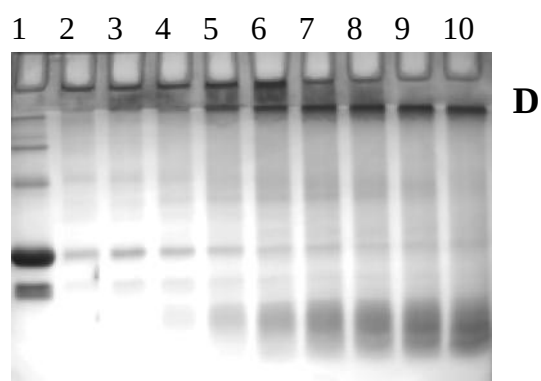
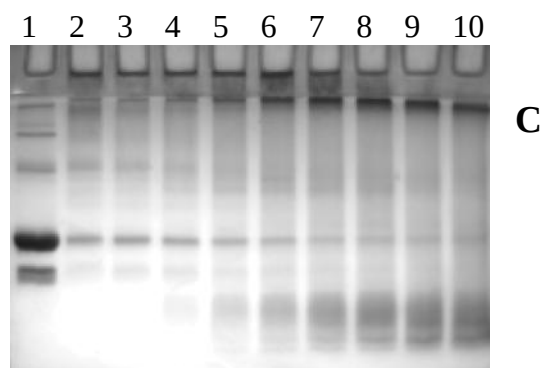
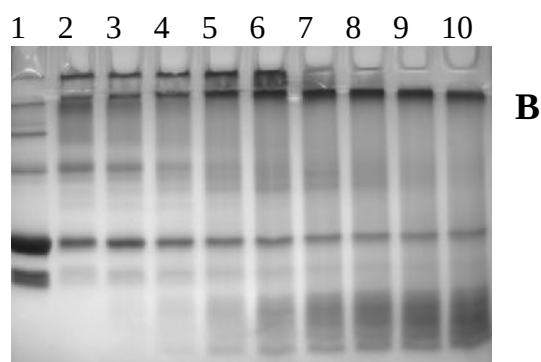
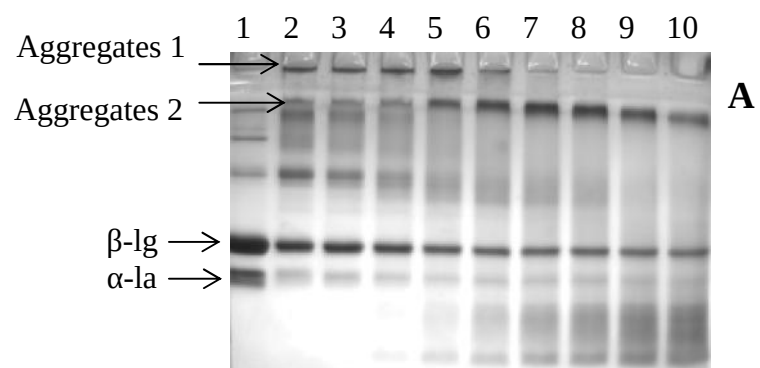
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.08.076>.

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Supplementary figure 1.



Supplementary figure 2.

